

Examination Of Transient Transfection As A Potential Means Of Recombinant Protein Production.

A PhD thesis submitted to University College London by

Andrew En-Tzu WU

Department Of Biochemical Engineering
University College London
Torrington Place
London
WC1E 7JE

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*All this I tested by wisdom and I said ,
"I am determined to be wise" –
but this was beyond me.*

Ecclesiastes 7:23

Abstract

The application of transient transfection to the production of recombinant proteins in mammalian cell culture is examined in this thesis. Transient transfection is able to rapidly produce significant amounts of recombinant protein in a matter of weeks. This allows the early product development, such as preclinical testing and characterization, to be performed independently of the lengthy cloning process that can take up to 6 months. Selection of cell line (CHO), culture conditions (serum-free) and transfection agent (polyethylenimine) were chosen with a focus on industrial relevance. Physicochemical characterisation of PEI-DNA complexes formed in physiological buffer (150 mM NaCl) and culture media (DMEM and CHO-S-SFM II) showed that positive zeta potential during formation lead to higher transfection efficiency. Particle studies showed that initial aggregation during formation was reversed when complexes were diluted in media during transfection. DNA uptake is influenced by culture media in which the transfection takes place. Complexes formed in 150 mM NaCl had the highest transfection efficiency. Examination of process variables showed that complexes prepared in 150 mM NaCl were less sensitive to variations in formation incubation time than complexes prepared in culture media. The presence of conditioned media was determined to be detrimental to transfection efficiency and introduced more variability. Studies carried out into the effect of the volume fraction of PEI-DNA complex solution showed that 10% v/v gave better transfection efficiency. Additions to agitated microwells showed a transition in mixing time and mixing regime between shaking speeds of 200-250 rpm, mixing times were observed to decrease by two orders of magnitude. This was further showed by observations from still images of high-speed camera footage of increased deformation in the liquid surface between these shaking speeds. Scale-up of transient transfection for recombinant protein production across three geometries (24-well plates, shake flasks and bioreactor) gave comparable SEAP expression levels of 2.4- 2.8 mg/l, thus showing that transient transfection is a scalable process. Microwells are, hence, a suitable platform for process development, and productivity is translatable to larger scales. Overall this work has determined that with adequate characterisation of process parameters, transient transfection is a rapid tool for the production of recombinant proteins.

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Chapter 1

1 Introduction

Transient transfection is a powerful technique by which milligram quantities of recombinant protein can be generated by mammalian cell expression systems in a matter of weeks; sufficient for the initial pre-clinical drug studies. Hence, the use of transient transfection is regarded as a potential means of speeding up the therapeutic protein development process (Wurm and Bernard, 1999; Wurm and Bernard, 2001). Transfection is a technique by which DNA encoding a recombinant protein of interest is introduced into mammalian cells. For the protein to be stably expressed, the gene of interest has to be integrated into the host chromosome. This is difficult to achieve and only occurs in less than 1% of transfected cells. This population of cells then has to be screened for stable integrants with various techniques, such as the use of a selectable marker (Zeyda et al, 1999). The selection of a stable clone is a long process that can take up to 6 months.

Transient transfection is increasingly being seen as a quick means to produce proteins in mammalian cells, without having to go through the time-consuming process of stable transfection and selection (Wurm and Bernard, 1999). Transient transfection involves the formation of transfection complexes with the transgene DNA and a transfection agent that condenses DNA in order to aid gene delivery to cells. Cells are first grown up to a sufficiently high density, after which complexes of DNA encoding the recombinant protein (often in the form of supercoiled plasmids) and a transfection agent are then introduced into the culture. These are taken up by the cells followed by the subsequent production of the protein encoded by the genes.

This work aims to investigate the potential application of transient transfection of industrially relevant mammalian cell lines at process scales as a means of initially generating milligram quantities of therapeutic proteins for pre-clinical studies. The scale up of such a technique is not a straightforward process with many interdependent conditions involved. The aim of this work is to fully characterize this technique at the microscale and use engineering tools to predict the performance of this technique at large-scale.

The cationic polymer PEI was chosen to be the gene delivery agent, and initial studies were undertaken to investigate the physicochemical characteristics of PEI-DNA complexes. The aim is to characterize PEI-DNA complexes with application at large-scale in mind, and to subsequently correlate the findings with transfection efficiency in an industrially relevant cell line.

During scale-up, a process has to be shown to be robust, and to do so the critical parameters have to be controlled. In order for transient transfection to be implemented at large scale, process variables that could impact the performance need to first be identified. The likely factors, which were seemingly unimportant at the laboratory scale such as the complex formation time, were investigated for their impact on the transfection efficiency of PEI-DNA complexes.

The scalability of transient transfection was investigated by application to microwells and scaling up to bioreactors. The application of microwells to cell culture processes is becoming increasingly popular. This platform shows great potential for the high

throughput investigation of several cell culture variables simultaneously. As such, transient transfection, with its numerous variables, is suited to the application of microwell plates as an investigative format. The aim is to investigate the use of microwells for application to transient transfection, and following that, to determine the scalability of transient transfection from microwells to bioreactor.

Chapter 2

2 Literature Review

2.1 Mammalian cell culture for the manufacture of biopharmaceuticals

The use of mammalian cells as an expression system for the production of recombinant proteins is well established in the biotechnology industry (Hu and Aunins, 1997; Kretzmer, 2002). The advantages of using a mammalian expression system are its ability to perform post-translation modifications, accurately fold proteins to form biologically active molecules and then secrete the protein of interest. These attributes make the production of therapeutic proteins (growth factors, cytokines, antibodies, etc) in mammalian cells an extremely attractive option compared with production in bacterial or other microbial systems. This is especially so if the biological activity of the protein is dependent on it being correctly glycosylated. Currently, there are several cell lines commonly used for the production of recombinant proteins for therapeutic use. These include Chinese Hamster Ovary (CHO) cells, Murine Myeloma (NS0), Baby Hamster Kidney (BHK) and Human Embryonic Kidney (HEK-293) (Hesse and Wagner, 2000).

The current methodology used in protein production is to first generate a stable cell line producing the therapeutic protein of interest via transfection and high-throughput screening, followed by production of initial material for pre-clinical characterization studies. Currently this is a process that could take up to 12 months (Wurm, 2004).

2.2 Gene transfer in animal cells

The cloning process involves the transfer of DNA encoding the protein of interest into an industrial cell line with the hope of stable genomic integration in order for high-producing clones to arise (Gopalkrishnan et al, 1999; Wurm, 2004). There are currently several diverse methods being employed for gene transfer. Although these methods vary in terms of their effectiveness, for the purposes of this work, only the methods that are most viable for implementation on a large-scale are considered.

The most commonly used method for the delivery of DNA to cells is lipofection or the use of cationic lipids for DNA transfer (Felgner et al, 1987; Mahato et al, 1997; Hoekstra et al, 2007a) *in vitro*. Though this technique has proved to be very effective for use in laboratory transfections, its cost is a major issue when considering transfection methods for application at large-scale, making it unfeasible. Of all the methods developed for transfection of mammalian cells, calcium phosphate co-precipitation (Jordan et al, 1996; Meissner et al, 2001), calcium chloride (Calcfection) precipitation (Jordan et al, 1996; Lindell et al, 2004) and polyethylenimine (PEI) (Boussif et al, 1995; Schlaeger and Christensen, 1999; Godbey et al, 1999b; Godbey et al, 2000; Tait et al, 2004; Lungwitz et al, 2005) have the most potential for use at large-scale. A major factor for their suitability is their cost-effectiveness, an important consideration when dealing with hundreds to thousands of litres of cells.

2.3 Transient transfection: A process development tool in biomanufacturing

Transfection of mammalian cells is an established technique for the development of stable animal cell clones for industrial recombinant protein production as described in

Section 2.1. The difference between transient and stable transfection is that the cells are not sorted and screened for stable integrants in transient transfection. Here, the expression of the protein is brought about by gene delivery to the cells in culture, and the protein is harvested at the end of the culture period, bypassing the lengthy selection process. As such, the requisite amounts of gene delivery agent and DNA are much larger, thus necessitating a cost-effective gene delivery agent. Transient transfection provides an avenue for protein glycosylation patterns in the different producer cell lines to be studied in parallel. Protein glycosylation is critical in determining the efficacy and half-life of a biotherapeutic in a patient (James et al, 1995; Jenkins et al, 1996). Transient transfection can hence be a very powerful decision making tool in its ability to aid drug companies in the selection of the most clinically suitable expression system to effect the greatest degree of drug confidence. Not only is transient transfection a means of producing recombinant proteins, but it also has potential application in the production of larger, more ordered biotherapeutics such as adeno-associated virus vectors (Park et al, 2006; Reed et al, 2006). Because of its potential for obtaining significant amounts of protein in a relatively short time span, transient transfection is increasingly being investigated for application to biopharmaceutical development (Wurm and Bernard, 1999; Girard et al, 2002; Pham et al, 2003; Geisse and Henke, 2005; Galbraith et al, 2006; Baldi et al, 2007).

2.4 Polyethylenimine: A cationic polymer with potential for transient transfection

Polyethylenimine (PEI) is a cationic polymer with a high positive-charge density. It is this high charge density that allows PEI to form nano-particulate complexes with DNA. In PEI, every third atom is amino nitrogen that has the potential to be

protonated. This gives the PEI molecule enormous buffering capacity (Tang and Szoka, 1997), and has been likened to a “proton sponge” (Boussif et al, 1995; Akinc et al, 2005). It has been strongly suggested that it is this property of PEI that is responsible for its DNA transfer ability in cells (Boussif et al, 1995; Kichler et al, 2001). PEI was found to protect the complexed DNA from degradation by DNAases (Moret et al, 2001). This is important to preserve the integrity of DNA in the subcellular compartments, such as the lysosome, after uptake in order for protein expression to occur. PEI can be found in both linear and branched forms. Studies have been carried out to attempt to characterize the complexes formed by plasmid DNA and PEI (Choosakoonkriang et al, 2003). The focus has been on investigating the uptake mechanism of the complexes into cells and their subsequent transport route in the intracellular space, leading to the eventual expression of the transgene (Pollard et al, 1998; Godbey et al, 1999d; Bieber et al, 2002). The main focus of these studies is the application of PEI as a possible nonviral vector for the delivery of DNA in gene therapy. Hence *in vivo* studies have also been carried out in conjunction, to determine the efficiency of transfer and level of reporter gene expression (Thomas et al, 2005).

PEI is a suitable DNA delivery agent for the application of transient transfection at the industrial scale. It is considerably cheaper than Lipofectamine, especially when hundreds to thousands of litres of cells are transfected at the industrial scale (Rosser et al, 2005). And as previously mentioned a system has to be cost-effective in order to be feasible at the industrial scale. Although calcium phosphate precipitation is also a widely used, cost-effective DNA delivery method, transfections in serum-free culture conditions considerably reduce its efficiency (Jordan et al, 1996) as compared to PEI-mediated transfections. PEI has been demonstrated to be an effective gene delivery

agent transient transfection (Schlaeger and Christensen, 1999; Galbraith et al, 2006; Tait et al, 2004), and hence is chosen for this investigation of transient transfection.

In this work, the objective is to study transient transfection for production of recombinant proteins. Hence the focus will be on the behaviour of the transfection complexes in serum-free suspension media commonly used with producer cell lines such as Chinese Hamster Ovary cells (CHO).

2.5 Characteristics of PEI-DNA complexes

PEI is able to change its ionization state over a pH range due to its protonable amino nitrogen atoms. This allows it to interact with the negatively charged phosphate backbone of DNA and leads to a condensation of the large hydrodynamic volume of DNA to form small complexes (Boussif et al, 1995). There are several interrelated parameters influencing the transfection of PEI-DNA complexes. These include PEI form (branched or linear) (Tang and Szoka, 1997; Wightman et al, 2001), the molecular weight of the PEI molecule, (Godbey et al, 1999c), DNA concentration (Schlaeger and Christensen, 1999), nitrogen to phosphate ratio, and size of the complexes (Ogris et al, 1998).

2.5.1 Forms of PEI

PEI can be found as either branched or linear polymers. The most commonly used is the branched 25 kDa PEI, although, studies both *in vivo* and *in vitro* suggested that this might not be the most efficient form for DNA delivery. The linear form of 25 kDa PEI is gaining increasing acceptance (Pham et al, 2003; Derouazi et al, 2004). The linear form of 25 kDa PEI has successfully been used to transfect CHO cells in a

serum-free environment. Transfections with 22kDa linear PEI and branched PEI-DNA complexes (Wightman et al, 2001) have showed that when the complexes were formed in salt containing buffer, the transfection efficiency of the linear 22kDa PEI complexes was generally greater. These studies were carried out on various murine cancer cell lines (melanoma and colon carcinoma) in the presence of 10% fetal calf serum. It has been observed that the presence of serum has a positive effect on gene transfer and expression (Durocher et al, 2002). However, when PEI was used to transfect serum-free suspension cultured HEK293 cells, it was observed that branched 25 kDa forms were the most effective (Schlaeger and Christensen, 1999).

2.5.2 PEI molecular weight

The 800 kDa PEI was first described for use in transfection (Boussif et al, 1995). Other optimization studies were later carried out using PEIs of different molecular weights in order to determine the most effective form (Schlaeger and Christensen, 1999; Godbey et al, 1999c). Transfections using PEI of molecular weights 800 kDa, 60 kDa, 25 kDa, 22 kDa, and 2 kDa were studied (Schlaeger and Christensen, 1999). As mentioned above, it was seen from the work with HEK293 cells, branched 25 kDa PEI gave the highest expression levels, a 4-5 fold increase over transfection with the 800 kDa polymer, though the reason for this is not well understood. A combination of 70 kDa and 10 kDa, large and small, PEIs have also been used to improve packing of the complexes in an attempt to increased transfection efficiencies (Godbey et al, 1999a).

2.5.3 Nitrogen:Phosphate ratio and DNA concentration

The electrostatic interactions between PEI and DNA, and the eventual size of the complexes is thought to be dependent on the ratio of the number of PEI amino nitrogen atoms and the number of DNA phosphate groups (the N/P ratio). Several studies have reported a broad range of optimal N/P ratios for transfection. For CHO cells, it was reported that a PEI:DNA w/w ratio of 2:1 gave the best transfection efficiency results, this corresponds to a N/P ratio of around 13 (Derouazi et al, 2004). When the 800 kDa PEI was investigated for HEK293 cells, the optimal N/P ratio was found to be 10-13.3 (Schlaeger and Christensen, 1999). Again, the optimal N/P ratio was found to be between 4.8 to 6 for *in vitro* transfections of a number of cancer cell lines (Wightman et al, 2001). This wide range of values could be due to the studies being carried out in a variety of different cell lines, and under different culture conditions. The formation of PEI-DNA complex has been investigated using two photon fluorescence correlation spectroscopy (Clamme et al, 2003a). TRITC (tetramethylrhodamine isothiocyanate)-labeled 25kDa branched PEI and rhodamine-labeled pGeneGrip plasmid (5.1 kDa) was used. It was found that at N/P ratios of 10, ~86% of PEI remained uncomplexed, and that each complex particle contains an average of 3.5 plasmids and thirty molecules of PEI. Calculation of the fractional occupancy of DNA revealed that ~10% of the PEI-DNA complex volume is occupied by DNA.

The optimization of DNA concentration for transient transfection with PEI was reported in several papers and found to vary from 0.1 µg/ml (Schlaeger and Christensen, 1999) to up to 2.5 µg/ml (Derouazi et al, 2004). Generally, a balance

between the DNA concentration and the N/P ratio has to be met for optimal transfection efficiency, which might vary according to the cell line used.

2.5.4 PEI-DNA complex size

The size of DNA complexes is thought to be a critical parameter in the efficiency of cellular uptake for *in vivo* delivery due to limitations in the vascular system and diffusion through tissues (Goula et al, 1998). The application of complexes in the cell culture is less constrained in that the complexes are simply added to the culture and come into contact with the cells with far less difficulty. It has also been suggested that the nuclear envelope in animal cells presents an obstacle to gene delivery due to the size of the nuclear pores (Brunner et al, 2002).

There have been several studies into the size of PEI-DNA complexes using various techniques such as laser light scattering (photon correlation spectroscopy) (Ogris et al, 1998) and electron microscopy (Tang and Szoka, 1997). A wide range of particle sizes have been measured for PEI-DNA complexes. In general, PEI-DNA complexes seem to aggregate in the presence of salt (Ogris et al, 1998; Wightman et al, 2001). When formed in salt-free conditions, particles sizes of between 40-300 nm have been measured. But aggregates of up to several microns in size have also been detected. The effect of PEI-DNA complex size on the gene expression was investigated using both transferrin-conjugated and unmodified 800kDa PEI (Ogris et al, 1998). A strong relationship between the PEI-DNA complex particle size and relative gene expression was found.

Scanning electron microscopy of complexes formed with 25 kDa branched PEI in 150mM NaCl revealed complexes between 20-40nm in diameter (Dunlap et al, 1997) at a low N/P ratio of 1.6. This is in contrast with the complex sizes of 200-500nm seen in other systems (Ogris et al, 1998). When the complexes were formed in HEPES buffered 150mM saline, aggregation of up to 1200nm was seen at lower N/P ratios, increasing the ratio to 6 or greater reversed this. But when the complexes were mixed in water or similarly lower ionic conditions (20mM HEPES buffer) no aggregation was seen. The size of the complexes ranged from 200nm at the highest N/P ratio of 8.4 to 1200nm at N/P ratio of 3.6. A possible explanation for this is that the larger sizes seen are the result of rapid aggregation of the smaller complexes when in the presence of salt containing buffer, as previously suggested. The mechanisms of PEI-DNA complex aggregation have been investigated (Sharma et al, 2005). Hydrophobic interactions seem to play a role in the complex aggregation; the use of surfactants has been attempted in order to stabilize the particle size. The aggregation also seems to be a polymolecular process, requiring collisions of primary particles in order to occur, increasing viscosity and a reduction in temperature seem to reduce this.

The size of PEI-DNA complexes appears to be strongly influenced by the charge ratio or N/P and also the DNA concentration.

2.6 Transfection of mammalian cells

As previously mentioned, there are many factors influencing the transfection efficiency of PEI-DNA complexes. Several studies have been carried out to correlate these factors with the actual transfection efficiency in different cell lines, and also the

reporter protein expression levels as an indication of effectiveness. A wide range of cell lines have been investigated for gene delivery with PEI-DNA complexes, which include a number of cancer cell lines (Wightman et al, 2001) with relevance to gene therapy applications, and also several industrial producer cell lines (Schlaeger et al, 2003; Muller et al, 2007; Sun et al, 2007).

Transient transfection of HEK293 cells expressing the SV40 large T-antigen or the Epstein-Barr Virus nuclear antigen protein was investigated (Durocher et al, 2002). These proteins are thought to increase expression of proteins in the cells (Shen et al, 1995). The HEK293 cells were grown on low-calcium hybridoma serum-free medium (HSFM) supplemented with 1% bovine calf serum. The transfections on cells cultured in fresh medium supplemented with 1% serum gave 4-5 fold increases in SEAP activity as compared with cells cultivated in serum-free medium. Furthermore, increasing the serum concentration up to 5% was reported to have further improved PEI-mediated transfection efficiency and protein production. There was no significant difference in SEAP expression when the transfection was carried out in medium conditioned for 24 hours. This indicates that medium exchange prior to transfection, as suggested elsewhere (E-J Schlaeger and K Christensen, 1999) is not required. This would significantly ease the scale-up of the technique, seeing that such a procedure would be difficult to accomplish at larger scales. Later work was performed on the possible use of protein hydrolysates as replacements for serum (Pham et al, 2003). The authors showed that while some peptones favour cell growth, they can be inhibitory to transfection. It was found that a gelatine peptone gave the highest level of reporter protein expression/activity and does not induce cell aggregation in contrast with other meat peptones.

Transient transfection has been widely performed in plate cultures of animal cells (Wightman et al, 2001; Breunig et al, 2004). The cells are exposed to the transfection media containing PEI-DNA complexes for up to 4 hours. The transfection media is then removed, and replaced with culture media. This is thought to minimize the cellular exposure to possible cytotoxic effects of PEI (Fischer et al, 1999; Breunig et al, 2004; Moghimi et al, 2005). It was also conversely found that in certain cases, the presence of serum is detrimental to transfection efficiency. This media change, between serum-free transfection media and serum-supplemented media, would also be a way of transfecting cell lines that are dependent on the presence of serum for growth.

Various events that occur during transient transfection in the cell are outlined in Figure 2.1. Each of these events, namely uptake and intracellular transport of PEI-DNA complexes, will be described in the next section.

2.7 Uptake and intracellular transport of PEI-DNA complexes

The attachment of PEI-DNA complexes onto the cell surface for endocytosis has been investigated. In order to elucidate the mechanism by which PEI aids the uptake of DNA into the cell, and the subsequent intracellular route, several studies have been carried out using fluorescently labelled DNA or PEI (Godbey et al, 1999d; Suh et al, 2003). Overall, the consensus is that cells take up PEI-DNA complexes by endocytosis (Klemm et al, 1998; Rejman et al, 2005; Breunig et al, 2007; Hoekstra et al, 2007b). It has been shown that PEI-DNA complexes attach onto discrete features of the cell plasma membrane and then migrate to common areas, such as coated pits

for cellular entry by endocytosis (Godbey et al, 1999d). The fluorescence seen on the inner surfaces of cytoplasmic vesicles 3 hours post-transfection gave evidence that the complexes have been taken up into the cell in vesicles such as endosomes or lysosomes, the inner surface of such vesicles being the cell exterior where the complexes attach on in the first place. Further studies have reported that electrostatic interaction of positively charged complexes with the negatively charged plasma membrane via two mechanisms; caveolae and clathrin-coated pits are the routes of cellular uptake of PEI-DNA complexes (Rejman et al, 2005). It has also been suggested that the PEI-DNA complexes tend to interact with glycosaminoglycans (GAG), which lead to inhibition of transgene expression (Ruponen et al, 2003; Ruponen et al, 2004).

The release of PEI-DNA complexes from endosomes has been attributed to the so-called “proton sponge” buffering capacity of PEI (Sonawane et al, 2003a; Akinc et al, 2005). Low pH in the endosome induced by the protonable state of PEI brings about an influx of chloride ions. This induces a secondary movement of water into the endosome, leading to swelling and consequent lysis, releasing PEI-DNA complexes into the cytosol.

The mechanism by which PEI-DNA complexes allow expression of the transgene has been heavily investigated. One possible mechanism is the interaction of PEI-DNA complexes with anionic phospholipids in the cell, which are synthesized for membrane regeneration. The negatively charged lipids could attach onto the positively charged surfaces of the complexes; the lipid groups then interacting with

the nuclear membrane, leading to eventual fusion and release of the complexes into the nucleus (Godbey et al, 1999d).

In order for expression to occur, the DNA must be unpacked from the complexes. Several methods have been suggested for the mechanism for the release of DNA from the complexes for expression. One method suggested that the rapid rate at which PEI-DNA complexes are transported into the cell, and the expression of reporter proteins 4 hours post-transfection have to be due to a more efficient mechanism other than random diffusion. The authors claim that the migration of complexes to the nucleus is due to active motor-protein driven transport through the cytoplasm on microtubules (Suh et al, 2003). Their studies showed that a large proportion of the PEI-DNA complexes were detected around the perinuclear region within 30 minutes post-transfection, suggesting that it is not transport to the nucleus that is the rate-limiting step. The rate limiting step could possibly be either endosomal release, nuclear uptake or vector unpacking. The nuclear pores can only reach a maximum size of ~33nm, which is too large for most of the particles to pass through. But the dissolution of the nuclear membrane during mitosis is an alternate means by which the complexes can enter the nuclear space (Grosse et al, 2006; Mannisto et al, 2007). Hence transfecting cells in the mid-exponential growth phase could possibly enhance efficiency and expression.

2.8 Large-scale transient transfection

There have been studies of transient transfection in bioreactors in an attempt to investigate scale up of recombinant protein production via this method (Schlaeger et al, 2003; Baldi et al, 2005; Galbraith et al, 2006). Recently, in addition to traditional

stirred tank reactors, transient transfection has been demonstrated to work in the Wave bioreactor system as well (Geisse and Henke, 2005). The most commonly used cell line for large-scale transfections is HEK 293. Transient transfection has been investigated for both HEK 293 cells (Baldi et al, 2005) and CHO cells up to the 100 l scale (Derouazi et al, 2004). These investigations have used the expression of model proteins such as monoclonal antibody IgG (Derouazi et al, 2004) and SEAP (Durocher et al, 2002) as an indication of transfection efficiency and hence likely protein productivities with this system.

2.9 Examining scalable transient transfection

2.9.1 Cell line

The aim of this work is to investigate the transient transfection process for recombinant protein production. For this purpose, it would be advantageous to select an industrially well accepted cell line. HEK 293 (Schlaeger and Christensen, 1999; Durocher et al, 2002; Wright et al, 2003) and CHO cells (Tait et al, 2004; Galbraith et al, 2006; Muller et al, 2007) have both been used for transient transfection with a view to large-scale application. In comparison with HEK 293 cells, CHO cells are better characterized and more extensively used in industry for the production of recombinant proteins (Andersen and Krummen, 2002; Butler, 2005). CHO cells have also been established for suspension culture in serum-free media. A combination of these factors makes it an attractive choice for investigation into the application of transient transfection at the process scale.

In line with the approach to industrial relevance, the use of serum-free culture media would also be preferred. The use of serum and other animal derived products presents

certain dangers, such as transmissible spongiform encephalites, to protein production for therapeutic applications (Merten, 1999). Because of this, the use of animal products is being phased out from industrial biomanufacture, and being replaced with serum-free and chemically defined culture media as the standard. Thus, a serum-free medium culture environment has been chosen for these series of studies into transient transfection.

2.9.2 Transfection agent

Polyethylenimine (PEI) was determined as the transfection agent of choice. As outlined in Section 2.4, PEI is a versatile delivery vector that is becoming increasingly popular for use in transient gene expression. Its relative low cost (as compared with cationic lipids) and comparatively high activity, without the need for additional modification, make PEI an attractive transfection agent. The most commonly used PEI is the branched form, of molecular weight 25 kDa (Schlaeger and Christensen, 1999; Wurm and Bernard, 2001). Previous studies have shown the use of 25kDa branched PEI in a CHO-S system with serum-free culture media (Tait et al, 2004). This work aims to further investigate its application to transient transfection by studying the characteristics of the complexes.

2.9.3 Plasmid DNA vector

Gene expression hinges not only on the transfection agent but also on the DNA vector introducing the transgene. Large-scale transient transfection would involve considerable amounts of DNA, in the region of milligrams to grams at a time, depending on scale. Because of this, the ease of DNA amplification is paramount. To

date, plasmid DNA is most commonly used for transient transfection due to its simple amplification in bacteria, and established purification techniques.

2.10 Overview of transient transfection

This discussion has examined the scope of transient transfection as a process with potential for the production of recombinant proteins. PEI has been shown in numerous studies to be an effective and versatile gene delivery agent across a variety of cell lines and culture systems. This thesis aims to investigate the application of transient transfection using PEI-DNA complexes with a view to process scale-up by first studying its physicochemical characteristics and subsequent correlation with transfection efficiency. Thus, the commercially accepted cell line CHO-S along with a serum-free culture environment was chosen as the model for studies into the scalability of transient transfection. Figure 2.1 outlines the keys steps in the procedure for transient transfection.

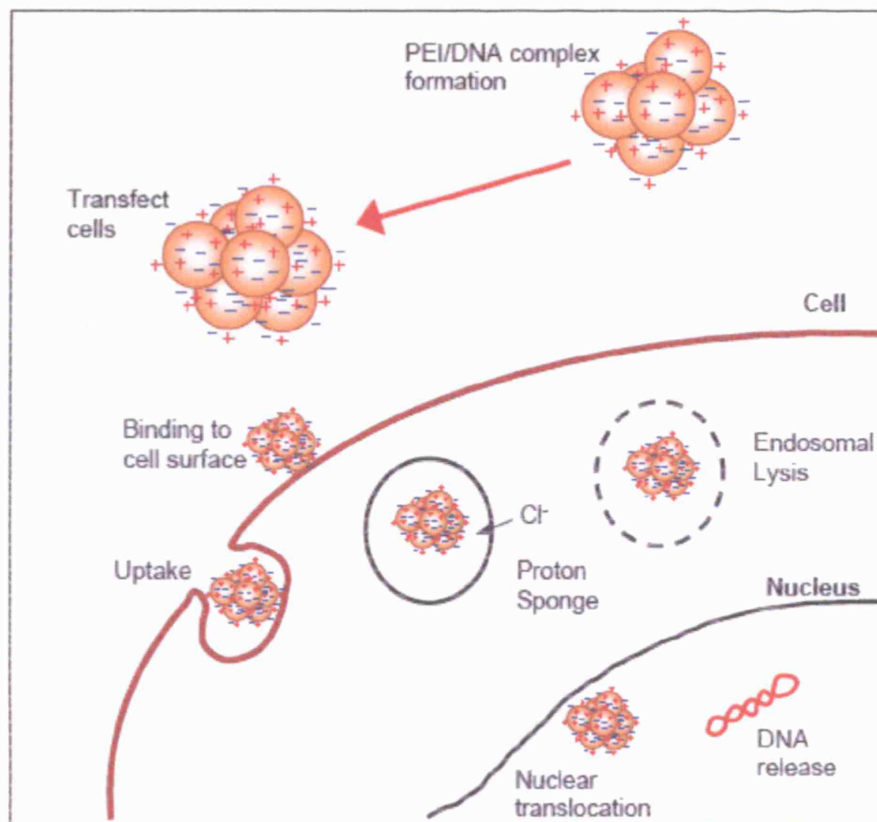


Figure 2.1 Schematic of key steps in transient transfection.

Chapter 3

3 Materials and Methods

3.1 Experimental procedures

3.1.1 General cell culture techniques

Suspension adapted CHO-S (Invitrogen, Paisley, UK) cells were grown in CHO-S-SFM II serum-free culture media (Invitrogen), in 125 ml or 250 ml disposable vent-capped Erlenmeyer shake flasks (Fisher Scientific, Loughborough, UK). The cells were maintained in a 37 °C, 5% CO₂ (v/v) Galaxy R CO₂ incubator (Wolf Laboratories, York, UK), on an orbital shaker (Ika, Germany) between 120-130 rpm. Cells were cultured between 2×10^5 cells/ml to 4×10^6 cells/ml, and passaged every 3-4 days up to a maximum of 20 passages.

A Master Cell Bank (MCB) and a Working Cell Bank (WCB) were created using the manufacturer's protocols (Gibco, 2003). The cryogenic storage medium was prepared using 50% v/v fresh CD CHO (Invitrogen) and 50% v/v conditioned CD-CHO media from 48 hours old cultures, DMSO (Sigma) was subsequently added to the mixture to a final concentration of 7.5% v/v. The storage medium was filter sterilized and stored at 4 °C until use. To prepare the cell banks, cells at the mid-exponential growth phase were centrifuged at 100× g for 5 minutes to pellet the cells. The supernatant was carefully aspirated, the cell pellet then gently resuspended in chilled cryogenic storage medium to a density of 1×10^7 cells/ml. The cell suspension was aliquoted into 1.8 ml Nunc Cryotubes (Fisher Scientific), containing 1.0 ml of cell suspension each. These

cryovials were then cooled at a controlled rate $-1\text{ }^{\circ}\text{C}/\text{min}$ of freezing. Cell banks were stored in liquid nitrogen dewars in the liquid phase.

After every 20 passage, a new vial from the WCB was thawed for culture. The vial was rapidly thawed in a $37\text{ }^{\circ}\text{C}$ water bath and then diluted drop-wise in cold CHO-S-SFM II media. The cells were centrifuged at $100\times g$ for 5 minutes, and the supernatant was aspirated to remove any DMSO. Finally, the cell pellet was gently resuspended in pre-warmed CHO-S-SFM II media and cultured in a 125 ml shake flask.

3.1.2 Plasmid DNA preparation and analysis

Plasmid gWIZ-GFP (Gene Therapy Systems, San Diego, CA, USA) encoding for green fluorescent protein (GFP) was kindly donated by GSK. This was amplified in *E. coli* DH1 cells grown in $2\times$ Terrific Broth (Sambrook and Russell, 01) in a 450 l pilot scale fermentor. The culture was harvested using a Carr Powerfuge tubular bowl centrifuge and the DH1 cell paste was stored at $-80\text{ }^{\circ}\text{C}$.

Plasmid gWIZ-SEAP encoding for Human Secreted Embryonic Alkaline Phosphatase (SEAP) was purchased from Gene Therapy Systems. The gWiz-SEAP plasmid was cloned into *Escherichia coli* DH5 α cells, and amplified in 2 l shake flasks in $2\times$ Terrific Broth. The *E. coli* cell suspension was harvested after 24 hours by centrifugation at $6000\times g$ for 15 minutes at $4\text{ }^{\circ}\text{C}$. DH5 α cell paste was stored at $-80\text{ }^{\circ}\text{C}$ until purified.

Both plasmids were purified using either Qiagen Maxi-Prep or Giga-Prep kits (Qiagen, Crawley, UK) depending upon the amount of plasmid required for particular experiments. The purification procedure was per the manufacturer's instructions (QIAGEN Plasmid purification handbook, 2005). Purified DNA was dissolved and stored in filter sterilised Tris-EDTA buffer pH 8.0.

Plasmid DNA concentration was determined based on absorbance at 260nm (A_{260}) using a BioMate 3 UV-Vis spectrophotometer (Thermo Scientific, Basingstoke, UK). A conversion factor of 1 absorbance unit of UV 260nm equal to 50 $\mu\text{g/ml}$ of double stranded DNA was used to convert A_{260} readings to concentrations (Sambrook and Russell, 01). Purified DNA was subsequently stored at $-20\text{ }^{\circ}\text{C}$ at 0.5 mg/ml. The purity of the plasmid DNA preparations was determined using the ratio of UV absorbances at 260nm/280nm ($A_{260/280}$) (Sambrook and Russell, 01) and also by 0.8% w/v agarose gel electrophoresis as described in Section 3.3.1. The purity of the DNA was quantified from the gel images using Quantity One 1-D Analysis Software (Bio-rad Laboratories, Hertfordshire, UK). Only DNA containing >90% supercoiled plasmid DNA was used for transfection experiments.

3.1.3 Polyethylenimine preparation

100% w/w Polyethylenimine (PEI) (25 kDa, branched, water-free) was obtained from Sigma (Dorset, UK). A stock solution of PEI (0.9 mg/ml) was prepared in ultrapure RO water and the pH adjusted to 7.2 with HCl. The PEI stock solution was sterilized by passing it through a sterile 0.22 μm filter (Nalgene, Hereford, UK) and was stored at room temperature until further use. All solutions were prepared using ultra-pure RO water (Millipore, Watford, UK).

3.1.4 Preparation of PEI-DNA complexes

The PEI-DNA complexes used throughout this work were formed at a (PEI) nitrogen : (DNA) phosphate ratio (N/P) of 10 (Schlaeger and Christensen, 1999; Schlaeger et al, 2003; Tait et al, 2004). This corresponded to a 1.39: 1 (w/w) ratio of PEI to DNA. Purified plasmid DNA at the stock concentration of 0.5 mg/ml was pre-diluted in half the final volume of the formation buffer to 20 µg/ml. The PEI required to complex the DNA at the chosen N/P ratio of 10 was also diluted in half the final volume of the formation buffer. These two stock solutions were then mixed and vortexed quickly to allow the complexes to form. The final concentration of DNA in the transfection solution was 10 µg/ml.

3.1.5 Shake flask transfections

Cells were transfected in 125 ml Erlenmeyer shake flasks with a working volume of 25 ml. CHO-S cells in the mid-logarithmic growth phase at 48 hours (Section 3.1.1) were pelleted by centrifugation at 100× g for 5 minutes. The cell pellet was then resuspended in fresh CHO-S-SFM II media to a cell density of 2.2×10^5 cells/ml. They were allowed to adapt for 1 hour in the cell culture incubator described in Section 3.1.1. PEI-DNA complexes were prepared in 10% v/v of the final culture volume (2.5 ml) as described previously (Section 3.1.4) in the respective formation buffer. After this, 22.5ml of cell suspension in each shake flask was transfected with the PEI-DNA complexes aseptically, giving a final culture volume of 25.0 ml and a final cell density of 2.0×10^5 cells/ml.

Samples (2 ml) were taken aseptically at intervals, the cell density and viability were determined by Trypan Blue exclusion assay, and a haemocytometer as described in Section 3.3.2. Cells counts were also performed using a CASY TTC automated cell counter (Innovatis, Reutlingen, Germany) as described in Section 3.3.2. The samples were centrifuged at $100\times g$ for 5 minutes, and the complexes in the supernatant were measured using a Zetasizer 3000 for particle size and particle count as described in Section 3.3.3. The complexes in the supernatant were also assayed using the heparin-mediated dissociation Picogreen quantification method described in Section 3.3.6 to determine the amount of DNA remaining in the supernatant.

3.1.6 Microwell plate transfections

All microwell scale transfections were carried out in 24 well ultra-low attachment (ULA) microwell plates (Corning Life Sciences, Netherlands). The plates were conditioned with CHO-S-SFM II medium prior to transfection. 1 ml of culture media was added to each well aseptically. The plate was then left to condition in the humidified CO₂ incubator at 37 °C for 1 hour. After conditioning, media in the plate was aspirated aseptically. The plates were then ready for transfection experiments. In parallel, cells for the inoculum were passaged into fresh CHO-S-SFM II media, and seeded into 125 ml Erlenmyer shake flasks at 2.2×10^5 cells/ml, and allowed to adapt for 1 hour in the incubator.

0.1 ml of PEI-DNA complex suspension, prepared as described in Section 3.1.4, were added to each conditioned well, followed by 0.9 ml of CHO-S cells from the adaptation shake flasks. The final working volume in each well was 1.0 ml at a cell density of 2.0×10^5 cells/ml. The plates were then aseptically sealed with Diversified

Biotech BreatheEasy membranes (Sigma) to maintain sterility, allow gas transfer, and minimize evaporation from the wells over the culture period (Zimmermann et al, 2003). The plates were incubated on a Heidolph Orbital Shaker (Fisher Scientific) at 120-250 rpm, in a humidified CO₂ incubator, set at 37 °C, 5% (v/v) CO₂. Various experiments were performed each with slight modifications to the transfection conditions. These are described later in the respective results chapters.

Samples were removed aseptically at intervals for analysis as with the shake flask experiments (Section 3.1.5). The film on each well was pierced with a 15-gauge hypodermic needle during sampling; the entire content of the well (~1 ml) was aspirated with a 1 ml pipette and used for analysis. Several wells were sampled at each time point to provide a mean and standard deviation for each measurement. Evaporation was monitored by weighing each sample in a pre-weighed microcentrifuge tube to determine the extent of water loss. All culture data was corrected for evaporation using the ratio between the initial weight (that is, 1 g) and the measured weight of the sample collected at each time point. Measurement of evaporation from the 24-well plates is described in Appendix A.

3.1.7 Stirred tank bioreactor transfections

3.1.7.1 Bioreactor description

Bioreactor transfections were carried out in a 5 l B.Braun BIOSTAT B-DCU stirred tank, unbaffled bioreactor (Figure 3.1). Agitation was provided by a single 3-blade pitched segment impeller (Marine type) with a diameter (D_i) = 0.07m. The impeller was set to downward flow. Downward flow of liquid would increase the residence time of bubbles, allowing more time for gas transfer (Sucker et al, 1994). The

impeller blades were set at a 45° angle to the axis of motion, with a clockwise rotation producing downward fluid motion (Figure 3.2). Aeration was achieved by pumping an air/nitrogen mix into the headspace of the bioreactor, and also through a porous micro-sparger at the bottom of the vessel, beneath the impeller. The bioreactor was connected to a B-DCU bench-top control tower with all supply modules, performing control of the agitation speed, temperature, pH and aeration rate. Data acquisition was carried out by the software MFCS/Win 2.0 via a linked PC. All equipment was purchased from Sartorius (Epsom, UK).

3.1.7.2 Bioreactor mixing time

Mixing times were measured in the 5 l stirred tank bioreactor (STR) with a 3 l working volume. The iodine and sodium thiosulphate decolourisation experiment described in Section 3.2.2 was used for the determination of mixing times for 1% additions. The mixing patterns were investigated using the high-speed camera and 1% v/v additions of blue food colouring tracer dye. Additions were made ~2 cm beneath the liquid surface within 2-3 seconds using a pipette via a port in the top plate.

3.1.7.3 Inoculum and other raw material preparation

One vial of CHO-S cells from the working cell bank (Section 3.1.1) was thawed in a 125 ml Erlenmyer shake flask containing 20 ml CHO-S-SFM II media. After 3 to 4 days of subculture expansion as described in Section 3.1.1, cells were pelleted to remove spent media by centrifugation, and expanded in 100 ml of fresh CHO-S-SFM II media in a 250 ml shake flasks. When the cell densities reached 2.0×10^6 cells/ml, cells were seeded into 2.70 l of pre-warmed CHO-S-SFM II media in the bioreactor at 2.2×10^5 cells/ml.

PEI-DNA complexes were aseptically formed in a 500 ml siliconized glass bottle according to the procedure described in Section 3.1.4. The 150mM NaCl complex formation buffer was sterilized using a 0.22 μm filter, then autoclaved. 150 ml of PEI 25 KDa, branched (27.8 $\mu\text{g/ml}$) was added to 150 ml of plasmid DNA (20 $\mu\text{g/ml}$) (purified as described in Section 3.1.2). The PEI-DNA complexes were mixed for 10 minutes using a sterilised magnetic stirrer bar. 300 ml of PEI-DNA complexes were then pumped into the bioreactor via STT quick sterile couplings (Sartorius). The final transfection cell density was 2.0×10^5 cells/ml and a plasmid DNA concentration of 1.0 $\mu\text{g/ml}$.

3.1.7.4 Experimental conditions

Bioreactor transfection experiments were carried out at a working volume of 3 l. The 5 l glass vessel was siliconized with Sigmacote (Sigma) prior to sterilization during the experiment set-up to minimize likelihood of the adhesion of cells or PEI-DNA complexes to the glass surface. The impeller speeds are described in the respective results sections. Temperature was set at 37 °C and controlled using an electrical heating blanket. The pH was set to 7.2; control was achieved using the bicarbonate buffering system in the CHO-S-SFM II medium by supplementing the inlet air supply with CO₂. A gas flowrate of 100 cm³/min was used in all experiments. The dissolved oxygen tension (DOT) was monitored by a polarographic oxygen electrode (Mettler-Toledo, Leicester, UK) and set at 30% \pm 1% of saturation. The DOT level was controlled by headspace aeration and also intermittent sparging of air and nitrogen. Each vessel was sterilized by autoclaving before each transfection. DOT electrode

calibration was performed each time before the bioreactor run using the “gassing-out” method with pure nitrogen and air.

Samples (7ml) were removed aseptically at intervals for analysis as with the shake flask experiments (Section 3.1.5).

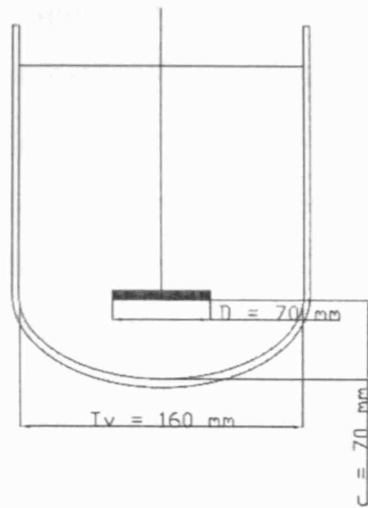


Figure 3.1. Schematic of 5 l stirred tank bioreactor.

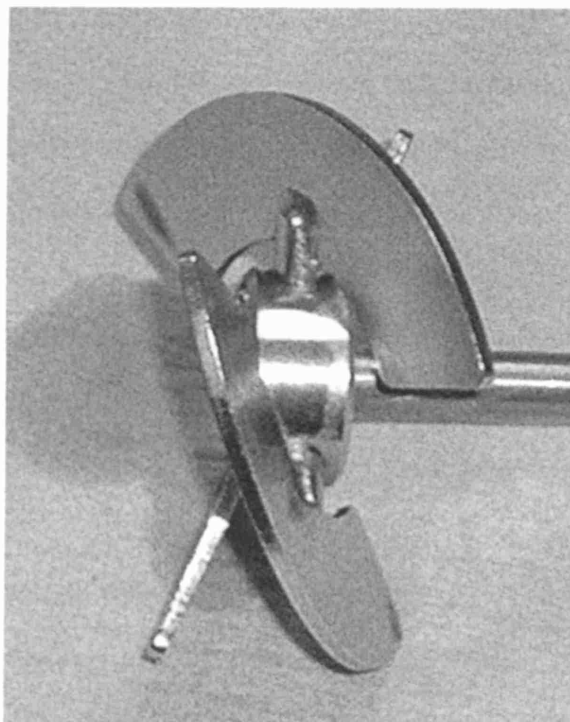


Figure 3.2. Segmented 3-blade impeller.

3.2 High speed camera mixing time measurements

3.2.1 Equipment set-up

High-speed videos were taken using a NAC HSV 500 digital high-speed video camera (NAC Image technology, Simi Valley, CA, USA) at 500 frames per second (fps) in order to determine the mixing regimes and mixing times for additions into 24 well microwell plates. The high-speed camera was kindly loaned from the EPSRC Equipment Loan Pool.

A single well model of the Corning Ultra-low Attachment 24 well microwell plate was fabricated in-house from Perspex and used for the video experiments as shown in Figure 3.4a. The model was designed with square faces to avoid distortion of the video images. For mixing time experiments the model was fixed onto a Heidolph orbital shaker (Fisher Scientific, Loughborough, UK) with a shaking diameter of 10 mm for experiments performed under agitated conditions. Addition of reagent was made possible through the use of a 10 μ l pipette tip attached to a 10 μ l pipette via flexible PEEK tubing as shown in Figure 3.3. This was held stationary above the well using a retort stand and clamps. The tip was held fixed over the center of the well using an expanded polystyrene cap fixed over the mouth of the microwell model. The flexible tubing allowed for the orbital rotation of the shaker platform whilst attaching the injection tip to the stationary pipette, providing ease of injection.

Mixing time experiments were also performed in a 5 l bioreactor as described in Section 3.17.2.

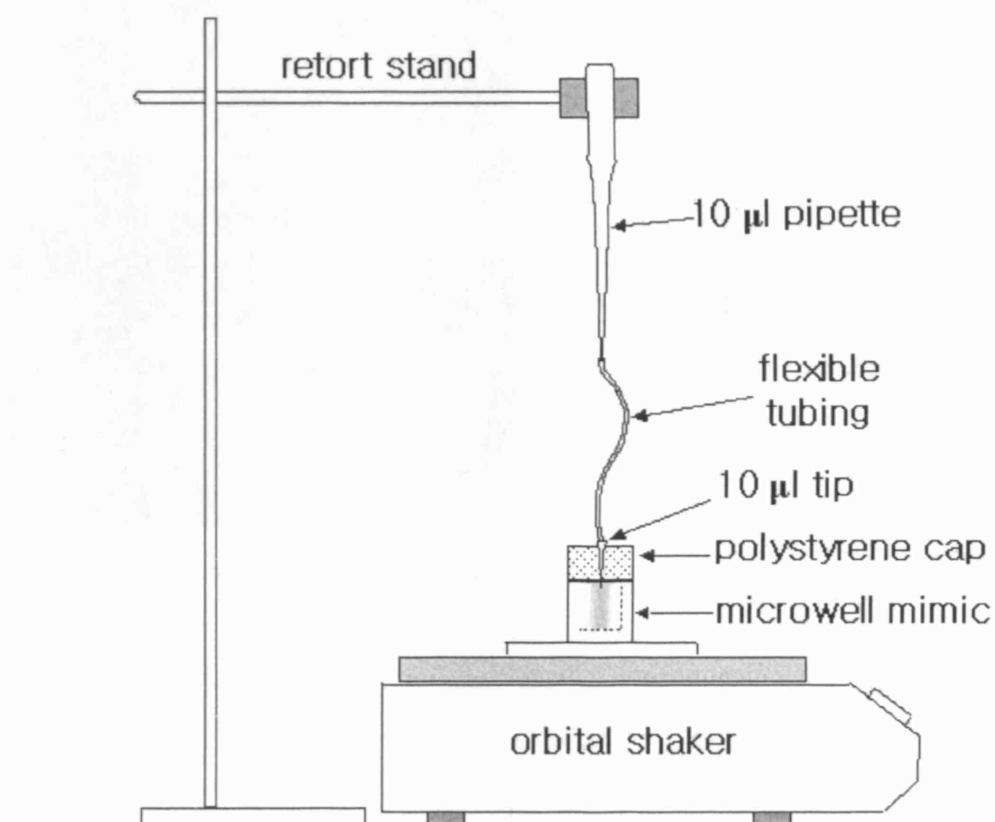


Figure 3.3. Schematic diagram of equipment used for high-speed video mixing time experiments.

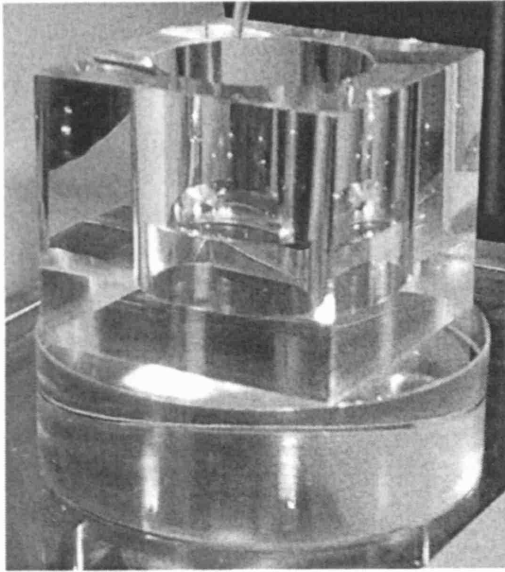


Figure 3.4a Perspex micro-well mimic.

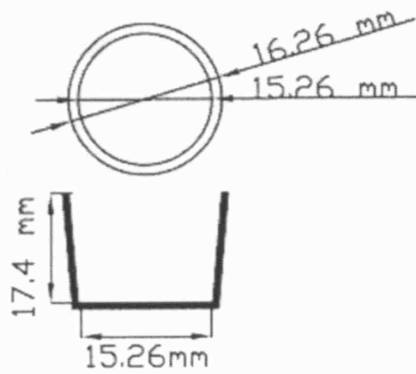
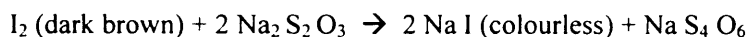


Figure 3.4b Dimensions of one well from 24-well ultra- attachment plate.

3.2.2 Decolourisation experiment

Mixing time was determined using the iodine and sodium thiosulphate decolourisation method (Bujalski et al, 1999). Iodine reacts quantitatively with sodium thiosulphate with a corresponding change in colour from dark brown to colourless as described below.



1.8 M sodium thiosulphate solution (Sigma) was added to 5 mM iodine solution (Sigma) at a ratio of 1:99 (v/v) in the microwell model and the bioreactor. The mixing time was determined to be the period between the point at which the thiosulphate solution has been completely added to the iodine, and the point when the iodine solution became completely colourless. Mixing times were determined by visual inspection and timing with a digital stopclock for mixing times longer than 10 seconds. The high-speed camera footage was used to determine rapid mixing times shorter than 10 seconds.

3.3 Analytical techniques

3.3.1 Agarose gel electrophoresis analysis of plasmid DNA

0.8% agarose gel was prepared using agarose powder (Sigma) and 0.5x Tris-Borate EDTA (TBE) buffer (Sambrook and Russell, 01). Ethidium bromide was added to the warm agarose solution. The plasmid DNA solution was diluted 1:1 with DNA gel loading buffer (Invitrogen) and loaded into each well. 0.5x TBE was also used as the

running buffer. Gels were run at 200 mA and 80 V constant voltage for 2 hours. Visualization was carried out after each run as described in Section 3.1.2.

3.3.2 Measurement of viable cell concentration

CHO-S cell density and viability were measured using the Trypan blue (Sigma) exclusion assay and the CASY TTC cell counter. For the Trypan blue assay, cells were diluted 2-10 times in 0.4% trypan blue solution (Sigma) depending on the cell density. 12 µl of diluted cell suspension was loaded into each chamber of a Neubaur haemocytometer (Fisher Scientific). A minimum of 100 cells was counted from each chamber. The CASY TTC (Sedna Scientific, Derbyshire UK) was also used for determination of cell counts and viability.

3.3.3 Size and particle count measurement of PEI-DNA complexes

The PEI-DNA complexes were prepared as described in Section 3.1.4 in 2 ml of the respective formation buffer. Samples, either freshly prepared or from transfection experiments, were measured using dynamic laser light scattering in a Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). The complexes were measured at every 10-15 minutes. At each time point, either 5 or 10 measurements were taken with a sampling time of 10 seconds. Temperature and agitation control of the formation conditions were achieved using an Eppendorf Thermomixer (Eppendorf, Cambridge, UK). The maximum coefficient of variance of the size measurements was 15% while that for the particle counts was 5%.

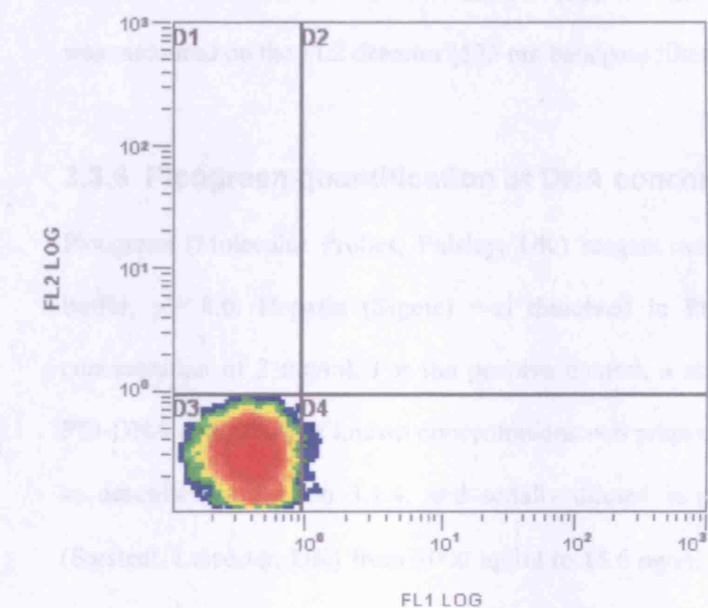
3.3.4 Zeta (ζ) potential measurement of PEI-DNA complexes

Complexes were prepared as described in Section 3.1.4 in 2 ml of the formation buffer. The ζ potential of either freshly prepared samples or those from transfection experiments was measured by injecting each sample into the ζ potential measurement chamber of the Zetasizer 3000 (Malvern Instruments). The complexes were measured at time intervals every 10 minutes. At each time point, either 5 or 10 measurements were taken with a sampling time of 20 seconds.

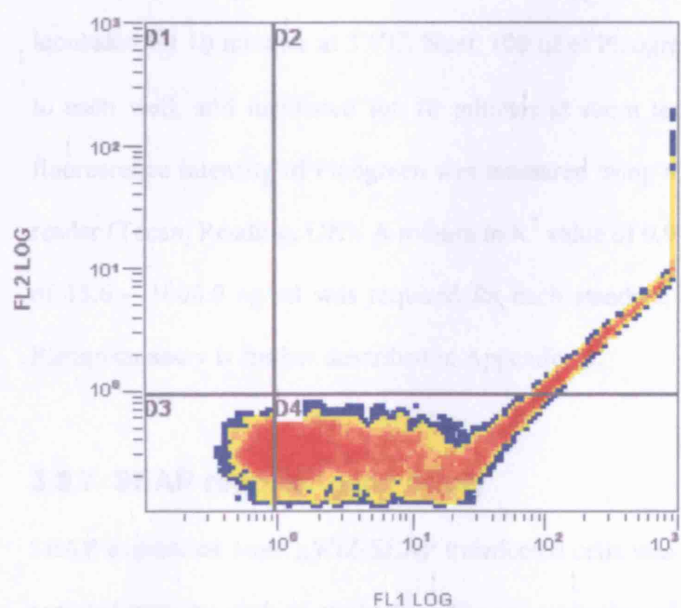
3.3.5 Flow cytometry for determination of transfection efficiency

The transfection efficiency is indicated by the percentage of viable cells in a population expressing GFP (Chu et al, 1999). GFP expression was determined using a Beckman Coulter Epics XL.MCL flow cytometer. Intact cells were first gated against debris. The GFP signal was then corrected against autofluorescence by only collecting fluorescence signals 98% greater than that from untransfected cells. Dead cells were excluded by staining with propidium iodide (PI) (Invitrogen) (Al Rubeai and Emery, 1993). Figure 3.5 shows typical flow cytometer measurement plots for untransfected negative controls and transfected CHO-S cells.

Samples from transfection experiments were centrifuged at $200\times g$ for 5 minutes, the cell pellet was then resuspended in cold, $\sim 4^{\circ}\text{C}$, $1\text{ }\mu\text{g/ml}$ PI stain in Phosphate Buffered Saline (PBS) solution pH 7.4 (Sigma). Cells were incubated on ice in the dark for 15 minutes to take up the stain, then centrifuged again as before and washed again in cold PBS to remove excess PI. The cell pellet was resuspended in 1 ml of cold PBS and then passed through the flow cytometer. 10,000 events were collected for each sample. GFP and PI dye were excited with a 488 nm argon laser, GFP



A



B

Figure 3.5 Example flow cytometry plots showing (A) untransfected cells and (B) transfected cells expressing GFP proteins. Plots are gated as follows: D1 Dead GFP negative cells, D2 Dead GFP positive cells, D3 Live GFP negative cells, D4 Live GFP positive cells.

emission was measured on the FL1 detector (525 nm bandpass filter), and PI emission was measured on the FL2 detector (575 nm bandpass filter).

3.3.6 Picogreen quantification of DNA concentration

Picogreen (Molecular Probes, Paisley, UK) reagent was diluted 150 times in TE buffer, pH 8.0. Heparin (Sigma) was dissolved in PBS, pH 7.4, at a working concentration of 3 mg/ml. For the positive control, a standard curve of dissociated PEI-DNA complexes of known concentrations was prepared. Complexes were formed as described in Section 3.1.4, and serially diluted in duplicate on 96-well plates (Sarstedt, Leicester, UK) from 1000 ng/ml to 15.6 ng/ml DNA, 100 μ l in each well. Samples from each time point of transfection experiments were added to each well in duplicate. Then 100 μ l of heparin 3 mg/ml was added to each well, and the plate was incubated for 10 minutes at 37 °C. Next, 100 μ l of Picogreen 150x dilution was added to each well, and incubated for 10 minutes at room temperature in the dark. The fluorescence intensity of Picogreen was measured using a Safire II fluorescence plate reader (Tecan, Reading, UK). A minimum R^2 value of 0.9 between the concentrations of 15.6 – 1000.0 ng/ml was required for each standard curve. Development of the Picogreen assay is further described in Appendix B.

3.3.7 SEAP reporter gene assay

SEAP expression from gWiZ-SEAP transfected cells was used as a reporter gene for secreted proteins such as antibodies. The concentration of SEAP in the culture media was determined using the chemi-luminescent SEAP Reporter Gene Assay kit (Roche Applied Science, Burgess Hill, UK). The optimal range for a linear standard curve was determined to be from 0-1000 ng/ml, showed in Figure 3.5. Samples were frozen

at -20 °C until assayed. The assay was then carried out according to the manufacturer's protocol. The maximum coefficient of variance for measurements was 15%.

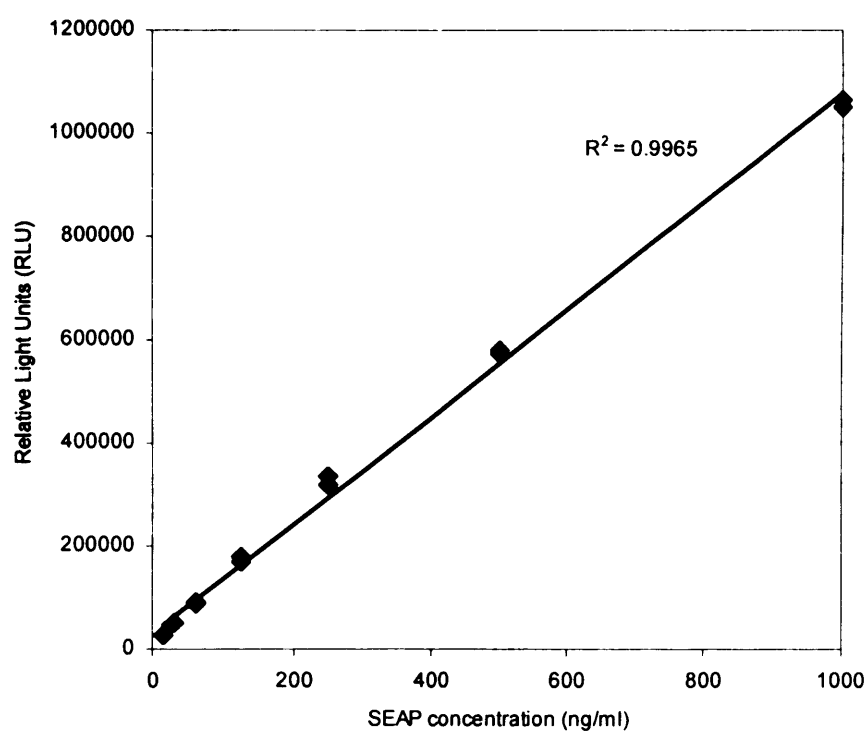


Figure 3.6 A typical standard curve for the SEAP chemi-luminescent assay.

Chapter 4

4 Physicochemical characteristics of PEI-DNA complexes and their impact on transfection

4.1 Introduction

In order for transient transfection to be applied to large-scale cell culture, it is critical that each of the various steps in the process be understood (Figure 2.1). One of the key variables is the choice of PEI and how it is employed within the process. Several PEIs have been reported in previous studies in terms of their gene transfer efficiency. These range from large 800 KDa PEIs (Boussif et al, 1995) to much lower molecular weight PEIs of 600 Da (Wightman et al, 2001). Various PEI Nitrogen : DNA Phosphate ratios (N/P) have also been investigated to determine the optimum N/P ratio for gene delivery (Schlaeger and Christensen, 1999). Several investigations into the optimal DNA concentration (Schlaeger and Christensen, 1999; Derouazi et al, 2004) have also been carried out. In general these studies have shown that transfection efficiencies over 70% GFP positive cells can be achieved with transfections of HEK293 cells (Durocher et al, 2002). Expression of the heterologous protein product occurring for up to 10 days during culture varying according to cell line and system (Schlaeger et al, 2003; Derouazi et al, 2004).

The size of the PEI-DNA complexes can affect cellular uptake and subsequent transfection efficiency in CHO-S cells (Ogris et al, 1998). In the large-scale transient transfection of mammalian cells, the PEI-DNA complexes should ideally be formed in growth media. This is such that on addition to the culture, the cells would not be

subject to a sudden change in osmotic conditions. Osmotic shock, due to the dilution of ionic concentration, can lead to damage of mammalian cells (Ho, 2006). This could possibly impair transfection efficiency and protein production. In this work, the 25 kDa branched PEI and a N/P ratio of 10 (which translates to a 1.39:1 PEI:DNA w/w ratio) as described by (Tait et al, 2004) was chosen as the model PEI of choice for the characterization work as outlined in Section 2.9.2. The DNA concentration of 1.0 µg/ml was chosen because it gave the highest transfection efficiency with a N/P ratio of 10. Here, the size of the complexes prepared in physiological buffer was investigated, as well as cell culture media for the purposes of transient transfection.

4.1.1 Aim and objectives

The aim of this chapter is to examine the physicochemical characteristics of PEI-DNA complexes during formation and to correlate them to the transfection efficiency of CHO-S cells. Hence the first step to be undertaken was to characterize the formation of PEI-DNA complexes for use in the transfection procedure. The effect of the observed characteristics of the PEI-DNA complexes on cells was subsequently tested in small-scale shake flask transfections.

The specific objectives are to:

- Establish methods for the rapid determination of the size/charge of PEI-DNA complexes.
- Determine the influence of the size and surface charge characteristics of PEI-DNA complexes on transfection efficiency.
- Correlate the physicochemical characteristics of PEI-DNA complexes with transfection efficiency in mammalian cell transfection.

4.2 PEI-DNA complex formation and characterization

Initial experiments focused on PEI-DNA complex formation and characterization in simple aqueous buffer. This had the advantage that all the components in the system were known and defined. The use of buffers also allowed for control of the physico-chemical environment such that the necessary analytical techniques could be established. The PEI-DNA complexes were prepared in a common buffer, 150mM NaCl pH 7.0 and two different types of culture media; standard mammalian cell media Dulbecco's Modified Eagle's Medium (DMEM), and a serum-free media CHO-S-SFM II, which contains protein hydrolysates. The complexes were formed as described in Section 3.1.4 at a nitrogen:phosphate ratio of 10 and immediately measured in the Zetasizer 3000 (as described in Section 3.3.3) for size and number of particles over a period of 60 minutes (Fig. 4.1a-d). Complexes were also prepared under agitated conditions to investigate if additional hydrodynamic shear had a significant effect on the size of complexes (Fig 4.1d). Complexes were first prepared then incubated at 500 rpm at room temperature (20 °C).

The results shown in Figure 4.1 indicate two distinct types of behaviour depending upon the composition of the medium in which the PEI-DNA complexes are dispersed. Increasing particle size over time was observed in the physiological buffers, along with a decrease in the particle count (Figure 4.1a). This indicates that aggregation of the individual PEI-DNA complex particles is occurring. When the complexes were formed in CHO-S-SFM II medium (Figure 4.1c), there was a sharp initial increase in complex size, reaching a maximum size of ~500nm after 40 minutes. This is contrasted by complexes formed in 150mM NaCl (Figure. 4.1a) and DMEM (Figure.

4.1b), where there was rapid increase in particle size over the period of 60 minutes, from ~ 300nm up to 1200 nm in the former and a similar trend of aggregation from 250 nm up to 1200 nm over 60 minutes for DMEM complexes. Compared with complexes formed in CHO-S-SFM II, there did not appear to be a plateau in the complex size after 60 minutes. The range of complex size between subsequent measurements was larger towards the later part of the incubation period for complexes formed in 150mM NaCl and DMEM.

There did not appear to be a significant difference in the particle sizes for complexes formed in CHO-S-SFM II medium under either static (Figure 4.1c) or agitated, at 500 rpm, conditions (Figure 4.1d). Early measurements had higher particle counts of 250 KCps for complexes formed under agitated conditions as compared with 100 KCps for initial measurements of statically formed complexes. However, any differences in particle counts or size rapidly disappeared.

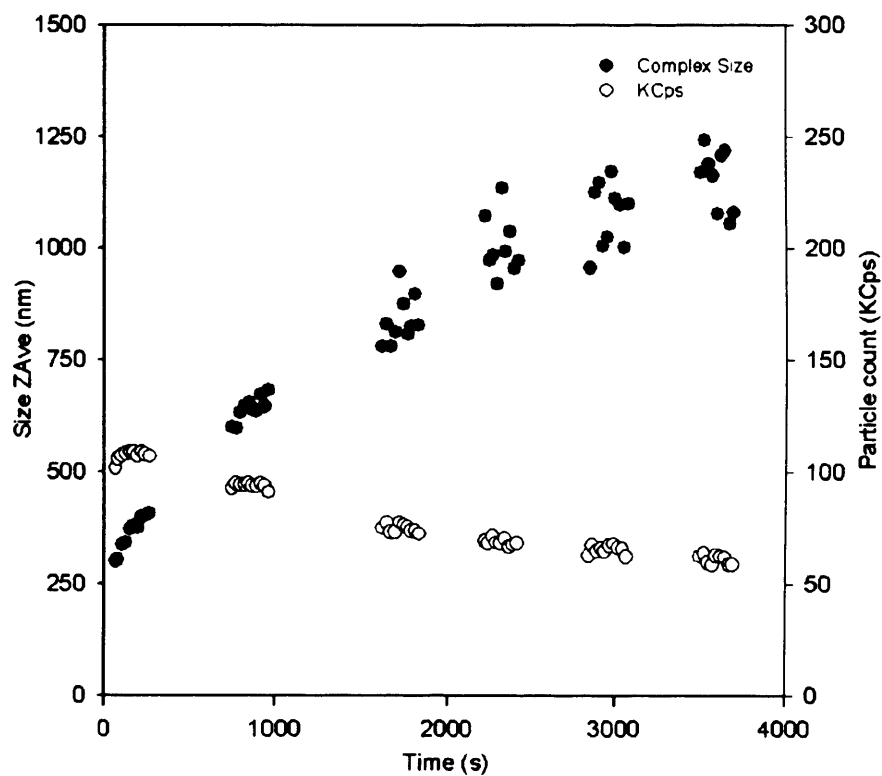


Figure 4.1a. Formation of PEI-DNA complexes in 150 mM NaCl aqueous buffer. Complexes were formed under static conditions, at room temperature with plasmid DNA gWiz-GFP and 25KDa branched PEI at a DNA concentration of 10 $\mu\text{g/ml}$ and an N/P ratio of 10 as described in Section 3.1.4. Samples were analysed in a Zetasizer over the course of 1 hour as described in Section 3.3.3.

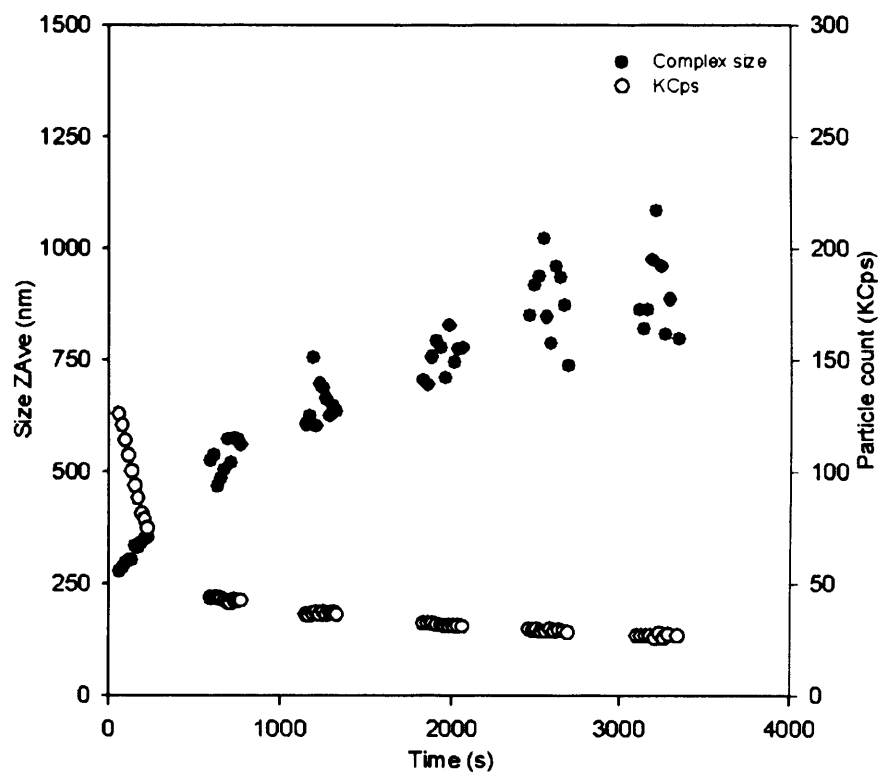


Figure 4.1b. Formation of PEI-DNA complexes in DMEM culture media. Complexes were formed under static conditions, at room temperature with plasmid DNA gWiz-GFP and 25KDa branched PEI at a DNA concentration of 10 $\mu\text{g}/\text{ml}$ and an N/P ratio of 10 as described in Section 3.1.4. Samples were analysed in a Zetasizer over the course of 1 hour as described in Section 3.3.3.

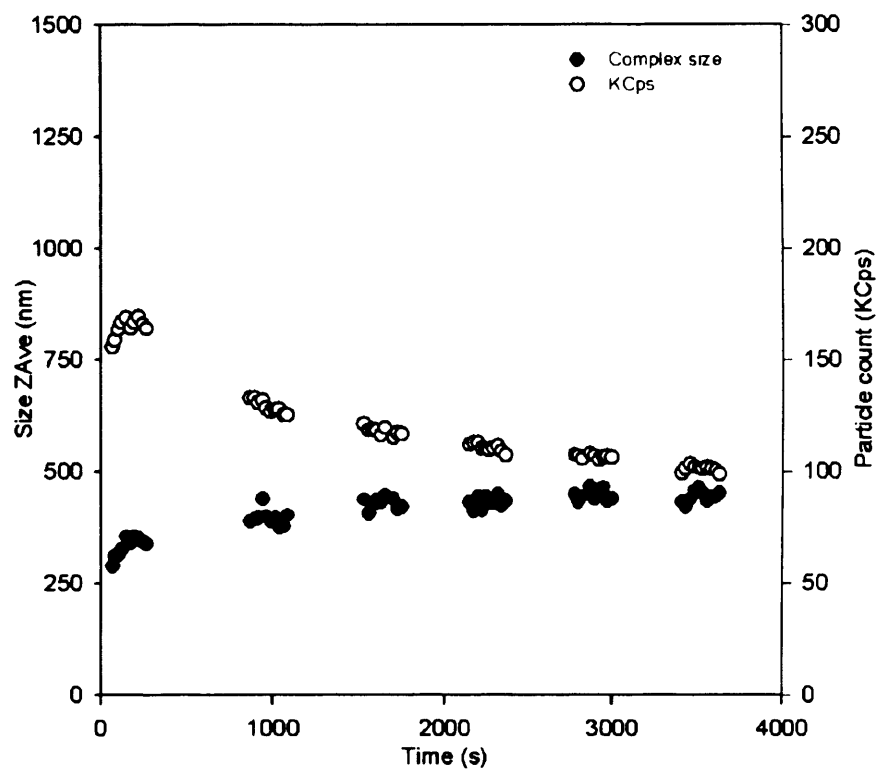


Figure 4.1c. Formation of PEI-DNA complexes in CHO-S-SFM II serum free growth medium. Complexes were formed under static conditions, at room temperature with plasmid DNA gWiz-GFP and 25KDa branched PEI at a DNA concentration of 10 $\mu\text{g/ml}$ and an N/P ratio of 10 as described in Section 3.1.4. Samples were analyzed in a Zetasizer over the course of 1 hour as described in Section 3.3.3.

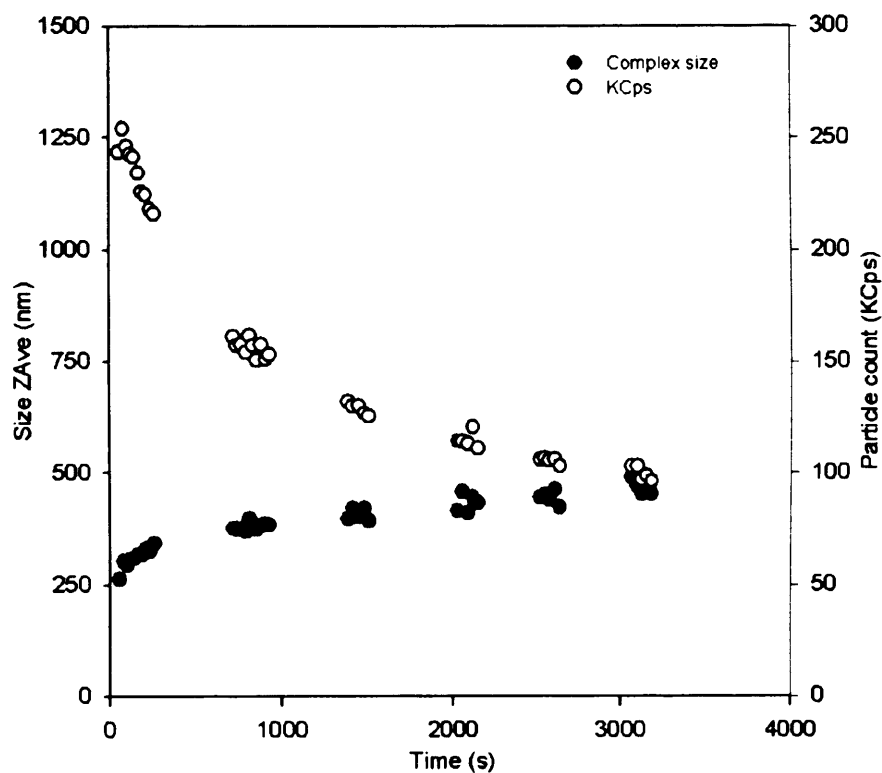


Figure 4.1d. Formation of PEI-DNA complexes in CHO-S-SFM II serum free growth medium under agitated conditions. Complexes were formed under agitated conditions of 500rpm in an Eppendorf Thermomixer, at room temperature with plasmid DNA gWiz-GFP and 25KDa branched PEI at a DNA concentration of 10 $\mu\text{g/ml}$ and an N/P ratio of 10 as described in Section 3.1.4. Samples were analyzed in a Zetasizer over the course of 1 hour as described in Section 3.3.3.

4.3 Dilution of PEI-DNA complexes in CHO-S-SFM II medium

During an actual transfection process, cells would come into contact with PEI-DNA complexes that (Figure 2.1) have been diluted in the culture medium after their initial formation. In order to investigate the effect this would have on the characteristics of the complexes, they were diluted 1:10 in pre-warmed CHO-S-SFM II media to simulate the ultimate process conditions.

Upon dilution of the complexes in CHO-S-SFM II media, there was a general decrease in the particle size down to ~200nm over the course of 60 minutes (Fig. 4.2a). The actual trend is difficult to discern due to scatter in the data obtained at the early time points. The decrease in size was, however, more rapid in the complexes formed in CHO-S-SFM II media than in the other solutions investigated. Particles formed in 150mM NaCl, then diluted in CHO-S-SFM II media had a much larger particle size distribution following initial dilution although the measured size then slowly decreased towards ~200 nm (Fig. 4.2b). It is interesting to note that this final particle size is very similar to that when complexes are first formed in CHO-S-SFM II. The change in particle size was less dramatic in complexes formed in DMEM. The early measurements show that the complexes were between 300-200 nm in size upon dilution (Fig. 4.2c). The particle size then decreased gradually over the course of 60 minutes to around 200nm.

Further measurements of particle size were carried out during transfection experiments (described in Section 4.5) over the culture period.

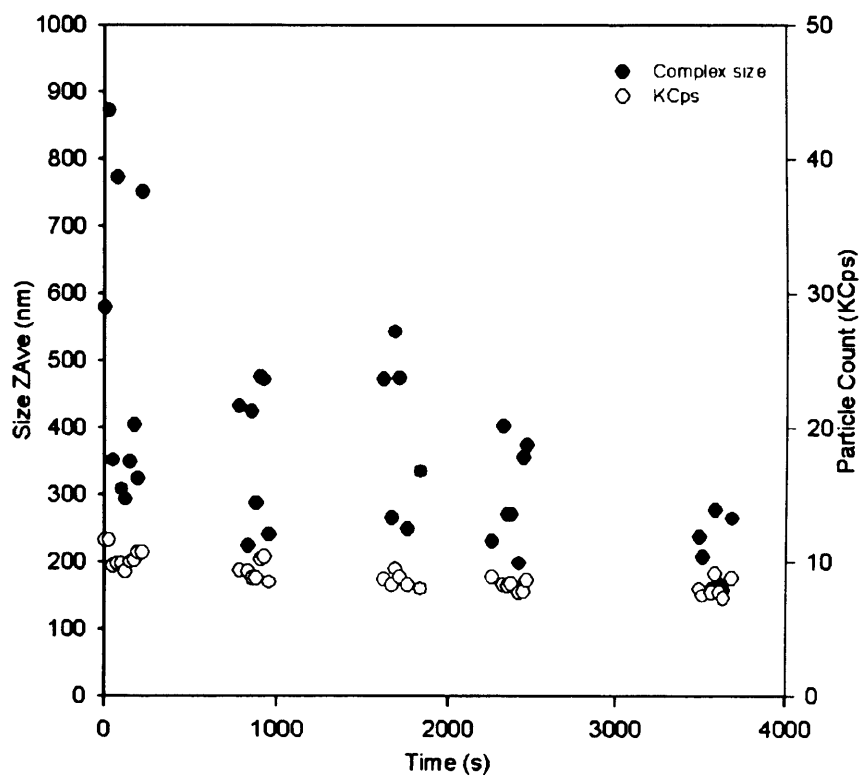


Figure 4.2a Dilution of PEI-DNA complexes formed in 150mM NaCl aqueous buffer 1/10 in CHO-S-SFM II media. Complexes formed were under static conditions at room temperature for 60 minutes as described in Section 3.1.4, then diluted 1/10 in pre-warmed CHO-S-SFM II media 37 °C. Samples were analyzed in a Zetasizer over the course of 1 hour as described in Section 3.3.3.

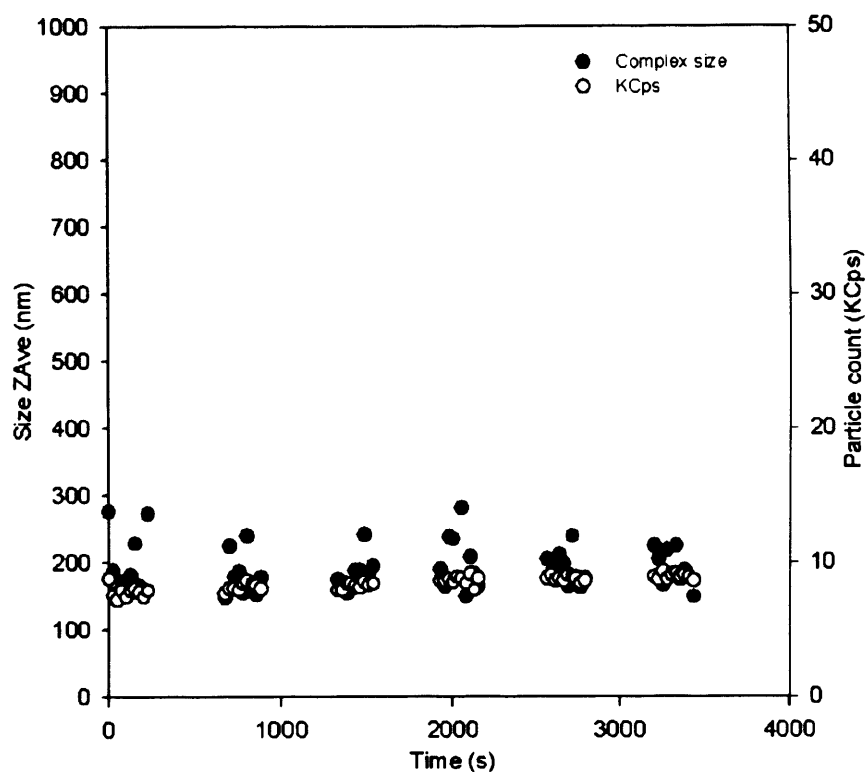


Figure 4.2b. Dilution of PEI-DNA complexes formed in DMEM culture media 1/10 in CHO-S-SFM II media. Complexes formed were under static conditions at room temperature for 60 minutes as described in Section 3.1.4, then diluted 1/10 in pre-warmed CHO-S-SFM II media 37 °C. Samples were analyzed in a Zetasizer over the course of 1 hour as described in Section 3.3.3.

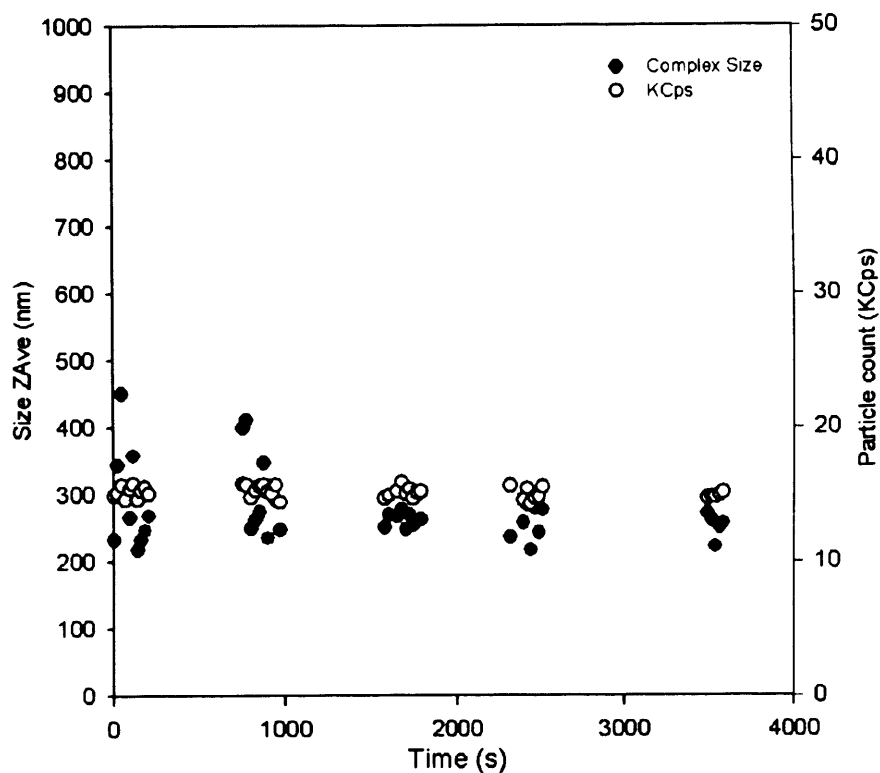


Figure. 4.2c. Dilution of PEI-DNA complexes formed CHO-S-SFM II, 1/10 in CHO-S-SFM II media. Complexes formed were under static conditions at room temperature for 60 minutes as described in Section 3.1.4, then diluted 1/10 in pre-warmed CHO-S-SFM II media 37 °C. Samples were analyzed in a Zetasizer over the course of 1 hour as described in Section 3.3.3.

4.4 Zeta potential measurements of PEI-DNA complexes

The ζ potential of complexes formed in various buffers was also studied based on PEI-DNA complexes diluted in CHO-S-SFM II medium after formation. It was seen that complexes formed in CHO-S-SFM II media exhibited largely neutral particle surface charge characteristics (Fig. 4.3). In contrast, complexes formed in NaCl solution had a much higher positive surface charge of +30mV while complexes formed in DMEM displayed strongly negative surface charge characteristics. In all cases however, once the complexes were diluted in culture media they all appeared largely neutral with only a slight negative charge.

4.5 Influence of PEI-DNA complex properties on CHO-S

Figure 4.3 Zeta potential measurements of PEI-DNA complexes

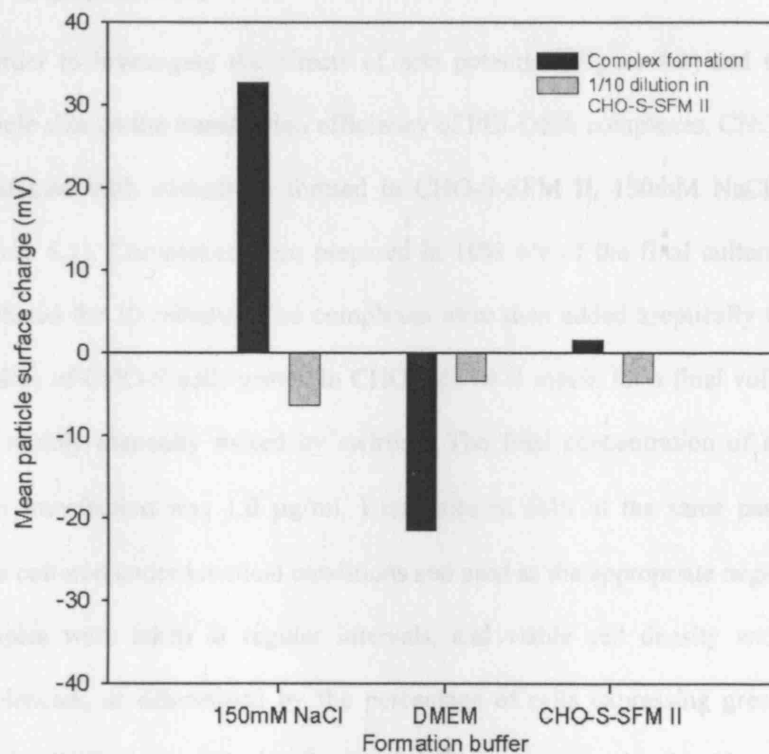


Figure 4.3 Zeta potential measurements of PEI-DNA complexes formed in various buffers (150 mM NaCl, DMEM, CHO-S-SFM II) followed by dilution in CHO-S-SFM II medium. Complexes were formed in a range of media as described in Section 3.1.4. Initial zeta potentials were then obtained using a Malvern Zetasizer 10 minutes after formation as described in Section 3.3.4. Samples incubated for 60 minutes during formation were subsequently diluted 1/10 in CHO-S-SFM II medium and the zeta potential measured again 10 minutes after dilution.

4.5 Influence of PEI-DNA complex properties on CHO-S transfection

In order to investigate the effects of zeta potential (Figure 4.3) and the change in particle size on the transfection efficiency of PEI-DNA complexes, CHO-S cells were transfected with complexes formed in CHO-S-SFM II, 150mM NaCl and DMEM (Figure 4.1). Complexes were prepared in 10% v/v of the final culture volume and incubated for 10 minutes. The complexes were then added aseptically to shake flask cultures of CHO-S cells grown in CHO-S-SFM II media for a final volume of 25 ml and rapidly manually mixed by swirling. The final concentration of plasmid DNA upon transfection was 1.0 µg/ml. Untransfected cells at the same passage number were cultured under identical conditions and used as the appropriate negative controls. Samples were taken at regular intervals, and viable cell density and transfection efficiencies, as determined by the percentage of cells expressing green fluorescent protein (GFP) (as described in Section 3.3.5) were measured at 24, 48 and 72 hours.

4.5.1 Cell growth and transfection efficiency

As shown in Figure 4.4, the cells transfected with complexes formed in culture media appeared to reach much higher cell densities at 96 hours as compared with those transfected with complexes formed in 150mM NaCl. The untransfected control shake flask cultures reached the highest cell density of 4.2×10^6 cells/ml at 72 hours. The transfection with 150mM NaCl complexes only reached 1.7×10^6 cells/ml at 96 hours.

Figure 4.5 shows the corresponding levels of GFP expression throughout each culture. The peak percentage of GFP expressing cells was observed at 48 hours in all cases. The cells transfected with 150mM NaCl showed the highest overall transfection

efficiency, 67% GFP positive cells at 48 hours. In all cases, the transfection efficiency began to decrease after 72 hours. When the viable cells were corrected for transfection efficiency to obtain the number of transfected (GFP positive) cells, the transfection with complexes formed in 150 mM NaCl showed the highest density of transfected cells at 72 hours of 3.92×10^5 cells/ml.

4.5.2 PEI-DNA Particle size and count measurements

In order to determine the effect transfection has on the PEI-DNA complexes, the particle size and count were measured. The variation in the number and size of PEI-DNA complexes in the culture supernatant during the course of the transfection experiments shown in Figure 4.4 were also investigated. This would also help to confirm the observations that were made when the transfections were simulated by diluting complexes in pre-warmed CHO-S-SFM II media as described earlier in Section 4.3. In order to analyse the PEI-DNA complexes remaining in the supernatant samples were centrifuged at $100 \times g$ for 5 minutes to pellet cells and obtain a cell-free supernatant.

As shown in Figure 4.6a the PEI-DNA complexes were initially of a similar size, 200-250 nm to those observed when diluted in CHO-S-SFM II in the absence of cells as described in Section 4.3. The complexes remained around this size range until after 36 hours, where there was a marked decrease in complex size to around 100 nm. In a few cases, such as transfections with complexes formed in 150mM NaCl, the complexes appeared to increase in size slightly up until 24 hours, after which it follows the trend of a size decrease from 36 hours.

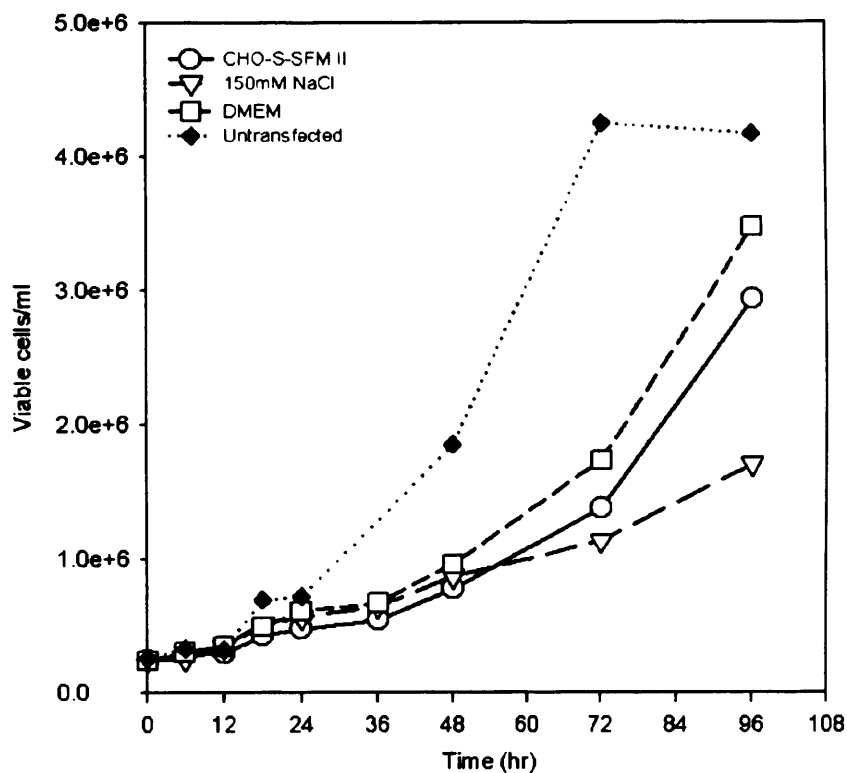


Figure 4.4 Growth kinetics of CHO-S cells cultured in CHO-S-SFM II culture media transfected with PEI-DNA complexes (1 $\mu\text{g}/\text{ml}$) prepared in various formation buffers. PEI-DNA complexes formed in either CHO-S-SFM II, 150mM NaCl or DMEM for 10 minutes as described in Section 3.1.4. CHO-S cells were cultured in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes added at time 0 hr. Viable cell counts performed as described in Section 3.3.2. Results show typical profiles from replicate experiments.

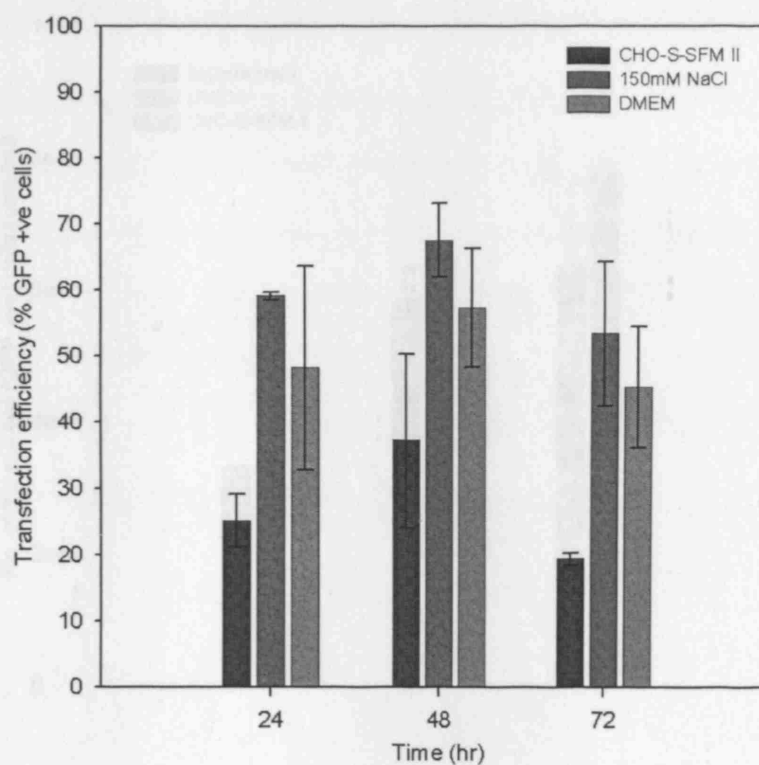


Figure 4.5 Transfection efficiency of CHO-S cells transfected with PEI-DNA complexes (1 μ g/ml) prepared in various formation buffer. PEI-DNA complexes formed in either CHO-S-SFM II, 150mM NaCl or DMEM for 10 minutes as described in Section 3.1.4. CHO-S cells were cultured in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes added at time 0 hr. The number of GFP expressing cells was determined by flow cytometry as described in Section 3.3.5. Results show the average of at least 3 measurements.

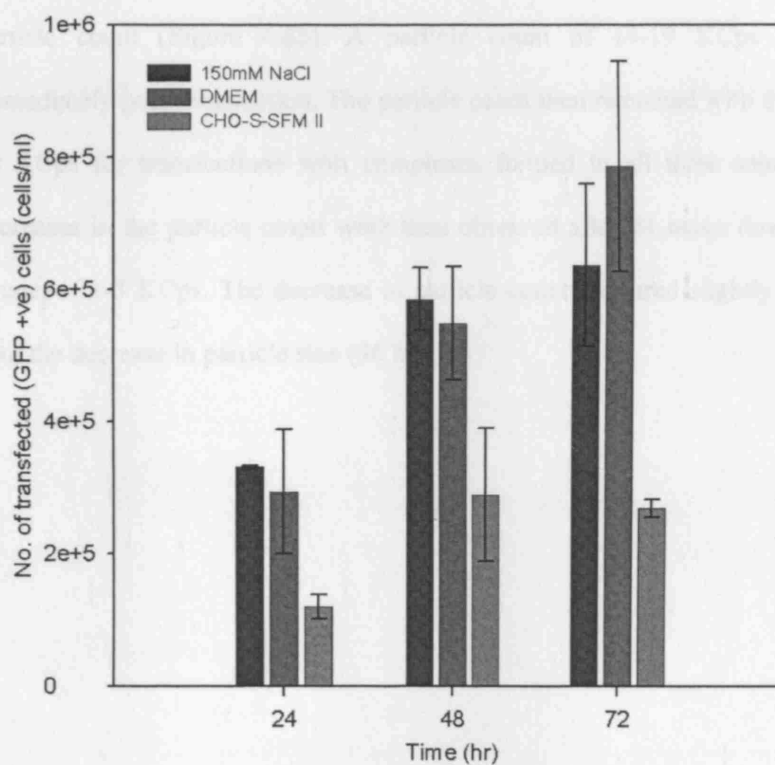


Figure 4.5a No. of GFP positive CHO-S cells transfected with PEI-DNA complexes (1 $\mu\text{g}/\text{ml}$) prepared in various formation buffer. PEI-DNA complexes formed in either CHO-S-SFM II, 150mM NaCl or DMEM for 10 minutes as described in Section 3.1.4. CHO-S cells were cultured in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes added at time 0 hr. The number of GFP expressing cells was determined by flow cytometry as described in Section 3.3.5. Results show the average of at least 3 measurements.

The trends seen in the measured particle size were mirrored in those of the complex particle count (Figure 4.6b). A particle count of 14-19 KCps was observed immediately post-transfection. The particle count then remained with the range of 14-20 KCps for transfections with complexes formed in all three solutions. Marked decreases in the particle count were then observed after 24 hours down to very low counts of 1-3 KCps. The decrease in particle count appeared slightly earlier (24 hr) than the decrease in particle size (36 hr).

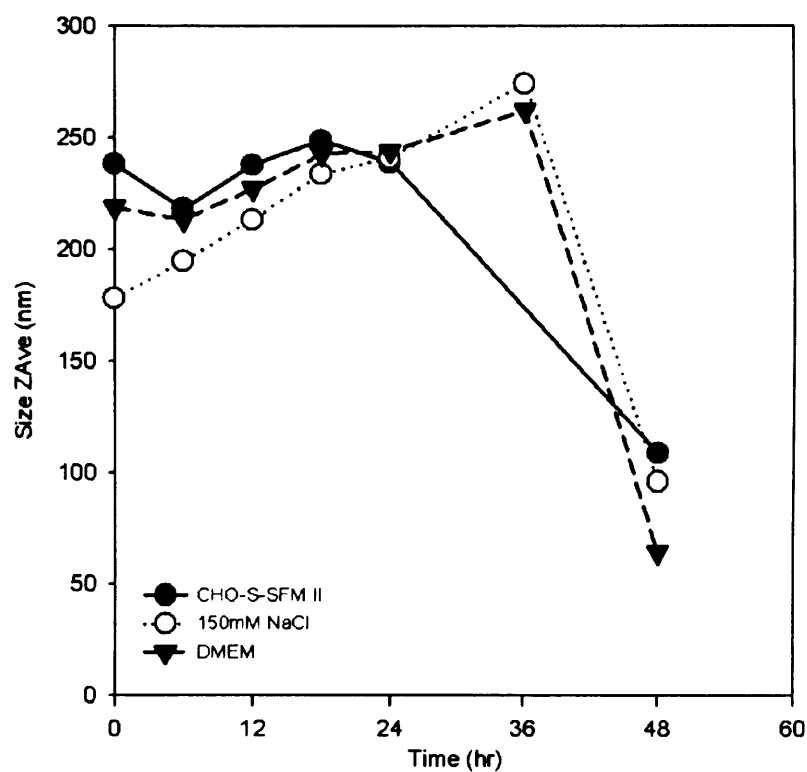


Figure 4.6a Change in PEI-DNA particle size post transfection. CHO-S cells cultured in CHO-S-SFM II culture media and transiently transfected with PEI-DNA complexes (1 $\mu\text{g}/\text{ml}$) prepared in CHO-S-SFM II, 150mM NaCl and DMEM as described in Figure 4.4. Complex size measured as described in Section 3.3.3.

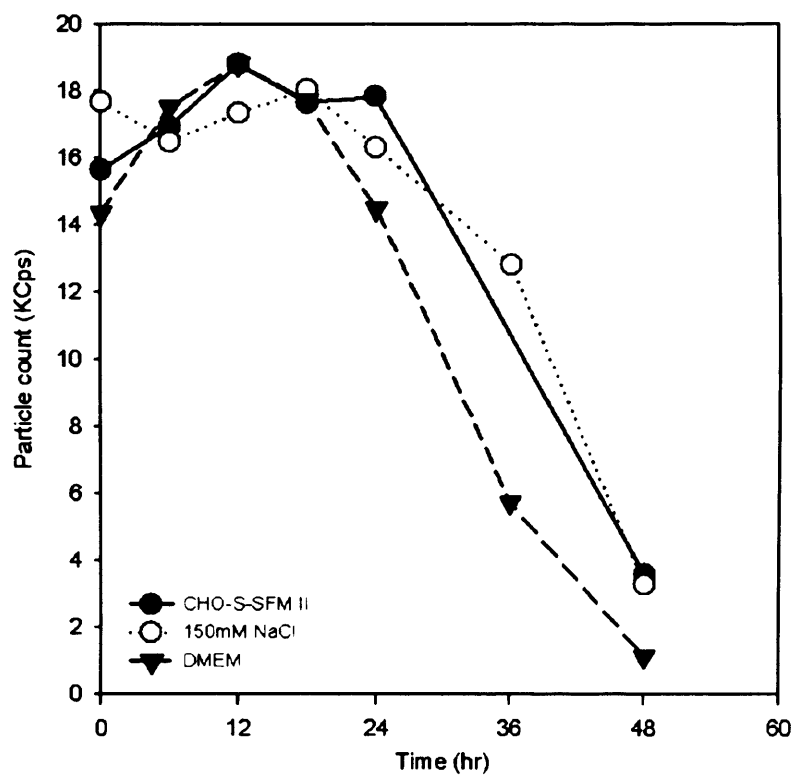


Figure 4.6b Change in PEI-DNA particle count post transfection. CHO-S cells cultured in CHO-S-SFM II culture media and transiently transfected with PEI-DNA complexes (1 $\mu\text{g}/\text{ml}$) prepared in CHO-S-SFM II, 150mM NaCl and DMEM as described in Figure 4.4. Complex size measured as described in Section 3.3.3

4.5.3 Uptake of plasmid DNA during transient transfection

In order to quantify the rate of uptake of DNA from the PEI-DNA complexes into the cells post transfection, a novel heparin based assay was developed as described in Section 3.3.6. Development of this assay is further described in Appendix B. Results showed an initial rapid uptake of DNA by the cells within 20 minutes. The concentration of DNA in the culture after transfection is 1000 ng/ml. (Figure 4.7) The earliest detectable reading immediately upon addition to the culture was much lower than the expected concentration of 1000 ng/ml. The concentration determined by the assay at 0 hr varies from 450 ng/ml, for complexes formed in CHO-S-SFM II, to 700 ng/ml for complexes formed in 150mM NaCl. In all transfections, the concentration remained around 500 ng/ml until 24 hours, before it decreased rapidly. This falls to below 100 ng/ml by 48 hours after transfection in all cases of shake flask transfections.

Initially, measurements made towards the end of the culture showed that the DNA concentration appeared to increase at 72 hours. This is most probably caused by cell death and the release of genomic DNA into the culture medium. In order to correct for this interference DNA concentration was measured in the supernatant of the sample without adding heparin. As DNA bound by PEI gives no Picogreen fluorescence signal, the fluorescence has to come from the lysed host cell genomic DNA (Appendix B). This concentration was then subtracted from the higher reading with heparin, giving the true concentration of plasmid DNA remaining in the supernatant as shown in Figure 4.7.

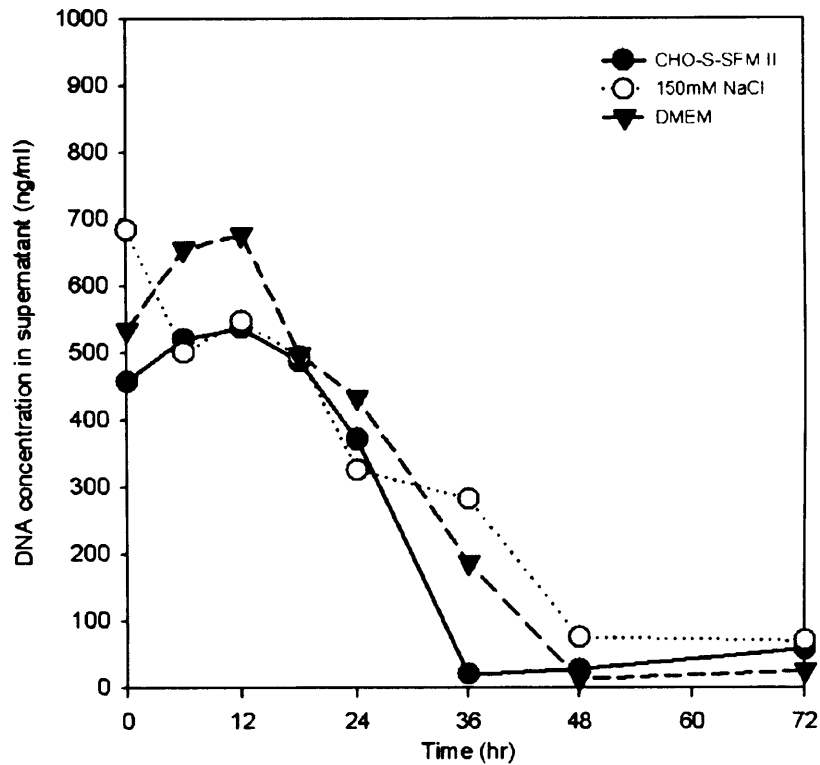


Figure. 4.7 Concentration of DNA remaining in the supernatant post-transfection of CHO-S cells cultured in CHO-S-SFM II culture media transfected with PEI-DNA complexes (1 $\mu\text{g/ml}$) prepared in various formation buffer. PEI-DNA complexes formed in either CHO-S-SFM II, 150mM NaCl or DMEM for 10 minutes as described in Section 3.1.4. CHO-S cells were cultured in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes added at time 0 hr. DNA concentration in the culture media was determined using with the Picogreen assay as described in Section 3.3.6. Results show averaged DNA concentrations from at least two replicate experiments.

4.6 Discussion

4.6.1 Complex formation

In order to fully understand the ways in which PEI-DNA complexes affect cells in culture during transfection and also to give insight into the possible factors influencing complex uptake, it was essential to first investigate the properties of the PEI-DNA complexes from a physicochemical perspective. The work described in this chapter has attempted to characterize the formation of PEI-DNA complexes formed in various common buffers in terms of their particle size and zeta potential. Further work was then carried out to relate the findings of the characterization stage with the behaviour of PEI-DNA complexes during and post the actual transfection of CHO-S cells in culture.

Initial characterization work was carried using a Malvern Zetasizer to measure the particle size and particle count of PEI-DNA complexes when formed in various aqueous environments such as culture media and salt solutions (Section 4.2). For all the systems investigated, an increase in particle size was accompanied by a corresponding decrease in particle count. It is suggested that the attraction between the cationic PEI and anionic DNA leads to very rapid formation of the complexes, and all available DNA is quickly bound up in the particles. The observed increase in particle size (eg. in Figure 4.1), is then most likely due to aggregation of the complexes rather than new complexes being formed or further adsorption of material onto existing complexes. The slower aggregation of complexes formed in CHO-S-SFM II (Figure 4.1c) suggests that the presence of protein hydrolysates in the medium is probably responsible for the slower rate of complex particle aggregation. The short

zwitterionic peptides in the hydrolysates, could possibly coat the surface of the complex particles, leading to decreased attraction between individual particles.

The initial rapid increase in particle size observed for example in Figure 4.1c could be due to the small primary complex particles undergoing aggregation from the interparticle interactions between the hydrophobic regions of the complexes formed in ionic buffer (Sharma et al, 2005). As these aggregates increase in size, the forces needed to draw them together would increase. There is also a decrease in available primary complex particles, as well as in the overall number of particles in the system, leading to fewer incidences of particle-particle collision during which aggregation can occur. Consequently, these factors lead to a slower rate of aggregation. The lack of these stabilizing hydrolysates in 150mM NaCl and DMEM media (Appendix C DMEM composition table) probably contributes to complexes formed in these media showing similar rapid aggregation profiles up to over 1000nm in size. The presence of salts also reduces the electrical repulsion between the particles. It has also been suggested that hydrophobic interactions rather than electrostatic interactions are primarily responsible for the aggregation of PEI-DNA complexes (Sharma et al, 2005).

4.6.2 Simulated transfection by dilution of complexes in pre-warmed CHO-S-SFM II media

To further simulate actual transfection conditions, the work described in Section 4.3 investigates the characteristics of PEI-DNA complexes when diluted in culture media. A probable reason for the complexes formed in 150mM NaCl (Figure 4.2a) taking longer to reach a steady state particle size as compared with those formed in CHO-S-

SFM II (Figure 4.2c) is the sudden change in the buffer environment. The presence of media components such as peptide groups would lead to a change in the surface charge characteristics of the complexes. This suggests that reassembly of PEI-DNA complex particles could be taking place. Complexes formed in CHO-S-SFM II would not have to undergo such readjustment, leading to their more rapid decrease to a smaller pseudo steady-state particle size. Surprisingly, the complexes formed in DMEM (Figure 4.2b) followed a profile more similar to that of those formed in CHO-S-SFM II upon dilution, as compared to complexes formed in 150mM NaCl (Figure 4.2a). As was postulated in Section 4.6.1, the absence of protein hydrolysates in DMEM and 150mM NaCl is what most likely lead to their similar complex formation and aggregation profiles.

The physico-chemical characteristics of DNA complexes after the transfection procedure have not been extensively characterized. Investigations are predominantly focused on characterizing the complexes during the formation stage (Ogris et al, 1998; Sharma et al, 2005; Akinc et al, 2005). From the simulated transfection experiments by diluting in CHO-S-SFM II media, there seems to be no significant difference in the size of the complexes that come into contact with the cells during transfection. All the complexes approached 200-250 nm in size. This therefore suggests that the size of the complexes that are eventually taken up into the cells is dependent only on the media in which the cells are cultured.

Another characteristic of the PEI-DNA complexes described in Section 4.4 that may influence their uptake by the cells is the surface charge density (Rejman et al, 2005). As shown in Figure 4.3, stronger surface charges were measured in complexes formed

in 150mM NaCl and DMEM than those formed in CHO-S-SFM II. Complexes formed in culture media, DMEM and CHO-S-SFM II both displayed negative ζ potential as compared with the complexes formed in 150mM NaCl. This is most probably because their compositions are more similar as compared with NaCl solution. The media constituents would contribute to charge neutralization of PEI. 150mM NaCl, on the other hand, would be less able to buffer the strong positive charge of PEI, leading to more positively charged ζ potential on the complexes. When diluted in CHO-S-SFM II to simulate transfection, all the complexes tended towards low negative surface charge (Figure 4.3). There was also a wider charge range post dilution measured for complexes formed in 150mM NaCl than those formed in DMEM and CHO-S-SFM II. This is in agreement with the previous hypothesis of an adjustment of complex particles formed in 150mM NaCl when diluted in media because of the sudden change in buffer environment. As the particles readjusted to the sudden addition of media components, its surface charge characteristics would change. This would lead to the wide distribution observed, as well as the slower stabilization of particle size as shown in Figure 4.2a. As the complexes formed in CHO-S-SFM II did not undergo such a change in the buffer environment, the charge distribution was much smaller. The trend towards a negative charge was probably due to the decrease in concentration of excess positively charged PEI from the effects of dilution. The low negative zeta potential observed in all the cases when the complexes were diluted in CHO-S-SFM II media is probably due to the media components, such as the protein hydrolysates as previously been suggested. These tend to be slightly acidic in nature, contributing to the observed zeta potential. It had been previously suggested that biological substances lead to greater particle stability than their zeta potential would suggest (Tipping, 1988). It is also possible that with a 10-fold dilution

in CHO-S-SFM II, the decrease in cationic PEI concentration leads to a shift towards neutral, as the available positive charge in the environment decreases. Once again, it appears that despite initial differences in charge characteristics during the formation of the complexes, once the complexes are diluted significantly in culture media, the predominating factor influencing the surface charge of the complexes during transfection is the culture media buffer environment, and these are the complexes which come into contact with the cells.

4.6.3 Shake flask transfection

In order to investigate the effects of zeta potential and the change in particle size on the transfection ability of PEI-DNA complexes, CHO-S cells were transfected in shake flasks as described in Section 3.1.5. A range of techniques to analyze both the cells and the PEI-DNA complexes were then applied to gain a greater understanding of the mechanism of the transfection process

As shown in Figure 4.4 the cell densities achieved in transfected cultures were much lower than those observed in the untransfected negative control. CHO-S cells are robust and commonly reach very high cell densities of 10^7 cells/ml (Hu and Aunins, 1997). Cultures transfected with complexes formed in DMEM reached a maximum cell density of 3.5×10^6 cells/ml. The highest transfection efficiency was observed in transfections with complexes formed in 150mM NaCl. In all three cases, the peak transfection efficiency was observed at 48 hours (Figure 4.5) post transfection, followed by a decrease in transfection efficiency at 72 hours.

A possible reason for lower cell densities observed in cells transfected with complexes formed in 150mM NaCl could be nutrient depletion due to higher transfection efficiencies (Figure 4.5). Under the strong CMV promoter, greater GFP expression could possibly lead to fewer nutrients available for biomass growth. The cells with lower transfection efficiency would have more nutrients available for cell growth, thus leading to higher observed cell densities. The endosomal lysis, in order for intracellular DNA release suggested by some, is also probably detrimental to trafficking of vesicles within the cell (Sonawane et al, 2003b; Akinc et al, 2005). A combination of these factors is most likely to have caused the reduced cell growth.

The corresponding DNA uptake was described in Section 4.5.3. As shown in Figure 4.7, a plateau in the uptake of plasmid DNA was observed between 0-24 hours. This might be due to the adsorption of PEI-DNA complexes to all available endocytosis uptake sites on the cell surface membrane (Godbey et al, 1999d; Rejman et al, 2005). Hence the initial concentration of 1000 ng/ml of DNA was not measured by the heparin-mediated dissociation Picogreen assay. Only upon cell growth or the renewal of the membrane binding sites does further uptake occur. This is seen as the further uptake and drop in DNA concentration in the supernatant coincides with the end of the lag phase at 24 hours or after a full cell cycle.

The plateau seen for both particle size (Figure 4.6a) and particle count (Figure 4.6b) is reflective of the plateau observed in the DNA uptake profile (Figure 4.7). As the particles attach on to the cell surface binding sites as previously suggested, no further uptake of complexes can occur due to saturation of available cell membrane endocytosis sites. Under these conditions the concentration of complexes present in

the media would remain fairly constant. In the short term, this would lead to the complexes reassembling and a smaller complex size as observed in the simulated transfections. The particle count would then be a function of this phenomenon. Up until 24 hours when the complex drops to around 100 nm, a slight increase in size was observed. This is probably due to aggregation of the unbound free complexes in the supernatant. Further DNA uptake after 24 hours (Figure 4.7) leads to a decrease in PEI-DNA complex concentration. This is probably what leads to a further decrease in the size of the complexes to ~100 nm at 48 hours and the particle count to drop from 14-19 KCps to 1-3 KCps. With a lower concentration of complexes in the system, the large PEI-DNA complex aggregates would be free to dissociate into smaller monomers as the propensity to aggregate is weaker. Another possible reason for this decrease in size is that the larger particles in the polydispersed size distribution of the complexes are selectively taken up into the cells, leaving the smaller particles.

All the complexes appear to have been taken up by the cells by 72 hours as shown in Figure 4.7. This would suggest even with the different conditions in which the complexes were formed in, the CHO-S cells have no trouble taking up the complexes. This is most probably due to the comparable surface potential characteristics of each of the different PEI-DNA complexes as indicated by the zeta potential measurements described in Section 4.4. It has been suggest that the binding of complexes to the cell surface membrane was related to the zeta potential of the complexes (Godbey et al, 1999a; Godbey et al, 1999d). Since the surface potential of the complexes is dependent on its immediate ionic environment, transfection in the same cell culture media would lead to similar complex surface charge characteristics in all three cases.

This would then lead to similar or closely similar uptake characteristics, and hence the profiles seen in Figure 4.7.

Finally, in Section 4.5.1 the impact of the PEI-DNA complex formation conditions on transfection efficiency was described. The decrease in the transfection efficiency at 72 hours, as shown in Figure 4.5, was most likely caused by dilution of the number of PEI-DNA complexes in each cell. Since all the complexes have been taken up by the cells, as shown in Figure 4.7, further cell division will lead to a dilution of the number of intracellular complexes. This would afterwards lead to a reduced number of cells able to express the GFP marker protein. Another possible reason for lower transfection efficiency is the degradation of plasmid DNA inside the cell (Lechardeur et al, 1999). It is most likely that a combination of these two factors is responsible for the observed decrease in transfection efficiency.

As there was no impedance to PEI-DNA complex uptake during transfection (Section 4.7), the difference in transfection efficiency between the three transfection conditions is most probably due to the zeta potential of the complexes during formation. As previously suggested (Section 4.6.2), the surface potentials of complexes in the three cases were similar (neutral) due to the media environment in which the transfections were carried out. It is then possible that the difference observed in the zeta potential of the complexes during the formation stage (Figure 4.3) is the critical factor. It has been suggested that the “proton sponge” effect of the PEI was responsible for the transfection ability of the PEI (Boussif et al, 1995) aiding the escape of PEI-DNA complexes from the endosomes after uptake, for transport to the nucleus (Godbey et al, 1999d; Akinc et al, 2005; Grosse et al, 2006). A mechanism by which this could

happen is by influx of ions into the endosome in order to balance the charges brought about by the complexes. This is followed by an influx of water in response to the osmotic pressure, leading to a rupture of the endosome, thus releasing the complexes into the cytosol (Sonawane et al, 2003b). The strong charge of complexes formed in 150mM NaCl and DMEM as observed during the formation (Figure 4.3) is probably responsible for this. As the complexes reassemble after transfection, the initial strong charges seen during formation appear to have been neutralized. A possible mechanism is that neutralization occurs on the surface of the complexes, with the interior of the complexes remaining at the previously high charge state measured during formation. Upon uptake into the cells, the high charge interior of the complexes formed in 150 mM NaCl induces the influx of ions to balance the sudden charge gradient within the endosome as described above. In the case of complexes formed in CHO-S-SFM II, the charges measured during formation were not as strong, and as a consequence, unable to induce the endosomal release of PEI-DNA complexes. The difference described here between complexes formed in different aqueous environments is most likely the reason for the difference in transfection efficiency observed.

4.7 Summary

The overall aim of this chapter was to investigate the physicochemical characteristics of PEI-DNA complexes, and relate this to transfection efficiency in CHO-S cells. The behavior of PEI-DNA complexes during formation stage strongly influences their performance during transfection. Initial particle sizes and zeta potentials during formation do not influence the uptake of the complexes during transfection. Rather, it is the culture medium in which the transfection takes place, which determines the uptake of complexes. Once diluted during transfection, the complexes readjust to the

new dispersant environment, acquiring similar surface charge characteristics. As such, future characterisation of complexes formed with PEI should include the studies into its physicochemical characteristics during the transfection step in order to accurately relate the behaviour of PEI-DNA complexes and transfection efficiency.

The zeta potential during the formation stage was found to be critical to the transfection efficiency of PEI-DNA complexes. It had been shown that the “proton sponge” effect mediated the escape of PEI-DNA complexes from the endosome by chloride accumulation and endosomal swelling (Sonawane et al, 2003b). The findings here suggest that strongly charged particles during the formation stage, as seen by the measured zeta potential, are more able to escape from the endosome, allowing for expression of the delivered genes. In order to determine the optimal formation buffer for transfections, the zeta potential of the complexes formed should first be measured (Section 4.4). This will give an indication as to whether the DNA in the complexes will be easily accessible; results implied high charge zeta potentials during the formation stage translate to higher transfection efficiencies.

Chapter 5

5 Impact of process related variables on transient transfection efficiency

5.1 *Introduction and Aim*

For transient transfection to be applied at the process scale, the critical process related variables have to be clearly defined. As a process increases in scale, many of the process parameters that were considered unimportant at the bench-scale become increasingly significant. Understanding the significance of these factors entails investigating the key process variables involved, and determining their effect on transfection efficiency.

Short complex formation times of 5-10 minutes are commonly used in transient transfection at the laboratory scale (Durocher et al, 2002; Clamme et al, 2003b; Derouazi et al, 2004). On a large-scale process such short times are not only difficult to achieve, but also unrealistic. The effect of longer formation times for PEI-DNA complexes on transfection efficiency has not previously investigated. Here, complexes formed in 150mM NaCl, DMEM and CHO-S-SFM II were studied for formation times of 10-60 minutes.

When a process is scaled up, it is also not always possible to separate large volumes of cells from spent conditioned media. This conditioned media contains a whole array of complex components such as host cell proteins and genomic DNA from dead cells (Alrubeai et al, 1995). It is still unclear what an effect such additional components

would have on the performance of PEI-DNA complexes during transient transfection. Most often, when scaling up to larger volumes, cells at high density are simply diluted several fold in fresh media (Hu and Aunins, 1997). For work described in this thesis so far, the cells have been centrifuged to remove the old, spent media, and seeded into fresh media before transfection (Section 4.5). When scaling up to larger bioreactor volumes, it is not always feasible to centrifuge the cells prior to inoculation. Hence, the effect of conditioned media carryover on the transfection efficiency of PEI-DNA complexes was investigated by adding media from 48 hours old culture into the shake flask transfections at 5% and 10% v/v. This was compared to a transfection with all the spent media removed.

In production scale bioreactors it would also be preferable to reduce the volume of PEI-DNA complexes for transfection. This would simplify the liquid handling. Transfection has been utilizing 10% of the final culture volume of PEI-DNA complexes. Reducing the volume of the PEI-DNA complexes would increase the amount of media present for cell growth and production. A decreased volume of PEI-DNA complexes would also likely lead to higher localized concentrations of PEI during transfection, which could lead to potential cytotoxic effects of PEI (Fischer et al, 2003). This chapter investigates whether forming a more concentrated mixture of PEI-DNA complexes in a smaller volume would affect the transfection efficiency.

5.1.1 Aim and objectives

The aim of this chapter is to assess the key process related variables involved in transient transfection using PEI-DNA complexes and how they might affect the

transfection efficiency of CHO-S cells. Particular emphasis will be based on the mechanism by which the transfection efficiency is influenced by measuring a range of system responses including cell growth, DNA uptake kinetics and particle size. The specific objectives of this chapter are to:

- Determine the influence of incubation times during PEI-DNA complex formation on transfection efficiency for complexes formed in 150mM NaCl, DMEM and CHO-S-SFM II. As described in Section 4.2 to Section 4.5, the formation media strongly influences the particle size during formation and also the zeta potential.
- Examine the carry-over of conditioned media from spent cultures during the inoculation step on transient transfection. As described in Section 5.1, removal of conditioned media during scale-up could be difficult and its effects on transfection efficiency were investigated.
- Determine the effect of reducing the volume fraction of PEI-DNA complexes on transfection efficiency. As described in Section 5.1, a reduction in the volume of PEI-DNA complexes could potentially be beneficial to transfection efficiency.

5.2 Influence of incubation time during complex formation on transfection efficiency

5.2.1 Cell growth

As described in Section 5.1, at the process scale it is not normally possible to consider operations of only a few minutes duration. Consequently, in this work, the influence

of complex formation times from 10 minutes to 60 minutes were investigated for their effect on transient transfection, following the studies carried out on the particle characteristics of the same time period as described in Section 4.2.

As shown in Figure 5.1, a similar trend in cell growth profiles was observed for transfections with complexes formed in 150mM NaCl (Figure 5.1a) and DMEM (Figure 5.1b), when the complexes were allowed to form for times between 10 and 60 minutes. Initially, there was no significant difference in the cell density up to 72 hours for both series of transfections, 150mM NaCl and DMEM. For transfections with 150mM NaCl complexes, complexes formed for 20 and 60 minutes transfections exhibited higher peak cell densities at 96 hours (Fig. 5.1b). This was followed by transfections with 30 minute, then 10 minute complexes achieving peak cell densities under 2×10^6 cells/ml.

There were much larger differences in the final cell density observed for transfections with complexes formed in DMEM (Figure 5.1c). The cell growth curves for CHO-S cells transfected with PEI-DNA complexes formed in DMEM (Figure 5.1b) showed a clear inflection point at 72 hours. The high cell density 96 hours measured from the transfection with complexes formed for 10 minutes could possibly be due to a measurement error. Cultures transfected with complexes incubated for 60 minutes and 20 minutes reached their highest cell density at 72 hours. Transfections with 30 minutes and 10 minutes complexes grew to the highest cell number at 96 hours, with the 10 minutes complexes transfection reaching the highest cell number of the four, at 3.5×10^6 cells/ml.

In the case of transfections with PEI-DNA complexes formed in CHO-S-SFM II, at the start there was no significant difference in the cell growth characteristics up to 48 hours (Figure 5.1c). This was no longer the case after 72 hours. Cells transfected with complexes formed for 60 minutes and complexes formed for 10 minutes reached the highest cell density of 3.0×10^6 cells/ml. This was followed by cells transfected with complexes formed for 30 minutes prior to transfection. Transfection with complexes formed for 20 minutes gave the lowest cell density at 96 hours, of only 1.5×10^6 cells/ml.

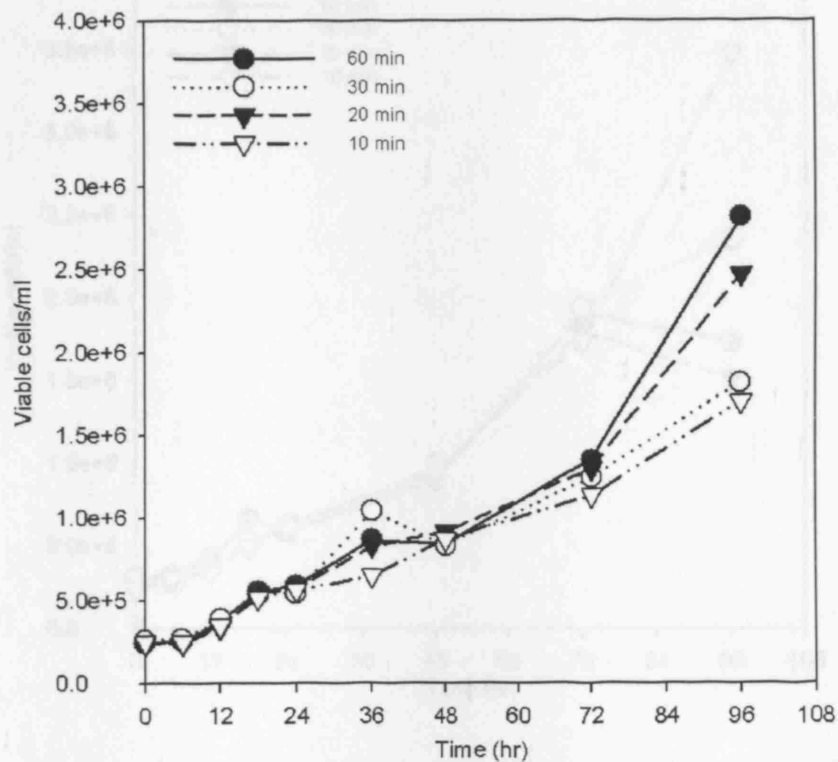


Figure 5.1a. Growth kinetics of CHO-S cells transfected with PEI-DNA complexes formed in 150 mM NaCl for 10-60 minutes. PEI-DNA complexes were formed in 150mM NaCl physiological buffer for 10, 20 30 and 60 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured on CHO-S-SFM II media in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes added at time 0 hr. Viable cell counts performed as described in Section 3.3.2. Results show typical profiles from at least two replicate experiments.

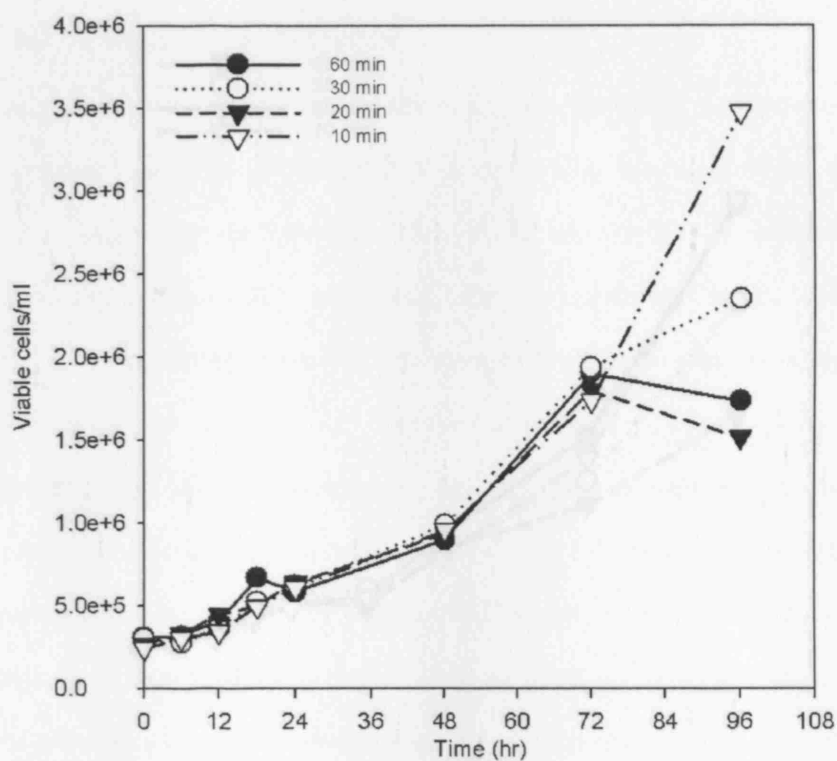


Figure 5.1b. Growth kinetics of CHO-S cells transfected with PEI-DNA complexes formed in DMEM for 10-60 minutes. PEI-DNA complexes were formed in DMEM basal culture media for 10, 20 30 and 60 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured on CHO-S-SFM II media in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes added at time 0 hr. Viable cell counts performed as described in Section 3.3.2. Results show typical profiles from at least two replicate experiments.

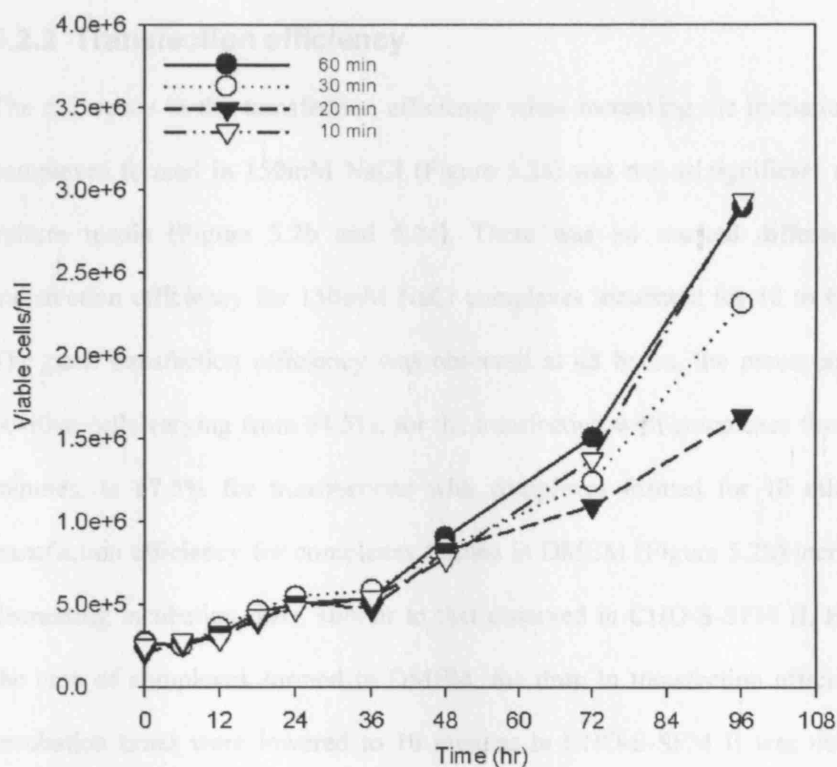


Figure 5.1c. Growth kinetics of CHO-S cells transfected with PEI-DNA complexes formed in CHO-S-SFM II for 10-60 minutes. PEI-DNA complexes were formed in CHO-S-SFM II culture media for 10, 20 30 and 60 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured on CHO-S-SFM II media in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes added at time 0 hr. Viable cell counts performed as described in Section 3.3.2. Results show typical profiles from at least two replicate experiments.

5.2.2 Transfection efficiency

The difference in the transfection efficiency when increasing the formation time for complexes formed in 150mM NaCl (Figure 5.2a) was not as significant as those in culture media (Figure 5.2b and 5.2c). There was no marked difference in the transfection efficiency for 150mM NaCl complexes incubated for 10 to 60 minutes. The peak transfection efficiency was observed at 48 hours, the percentage of GFP positive cells varying from 64.5%, for the transfection with complexes formed for 60 minutes, to 67.5% for transfections with complexes formed for 10 minutes. The transfection efficiency for complexes formed in DMEM (Figure 5.2b) increased with decreasing incubation time, similar to that observed in CHO-S-SFM II. However in the case of complexes formed in DMEM, the drop in transfection efficiency when incubation times were lowered to 10 minutes in CHO-S-SFM II was not seen, the proportion of GFP expressing cells continued to increase with shorter incubation times. The highest transfection efficiency was observed in cells transfected with complexes incubated for 10 minutes, with 47.9% GFP positive cells at 48 hours, 50% more GFP expressing cells than transfections with 60 minutes complexes at 32.0% transfection efficiency.

The transfection efficiency increased with shorter incubation times for the complexes formed in CHO-S-SFM II for 60, 30 and 20 minutes (Figure 5.2c). The highest transfection efficiency for CHO-S-SFM II complexes was observed in cells transfected with complexes incubated for 20 minutes at 43.8% GFP positive cells at 48 hours. A further decrease in the complex incubation time down to 10 minutes actually brought about a decrease in the transfection efficiency to 37.2% at 48 hours.

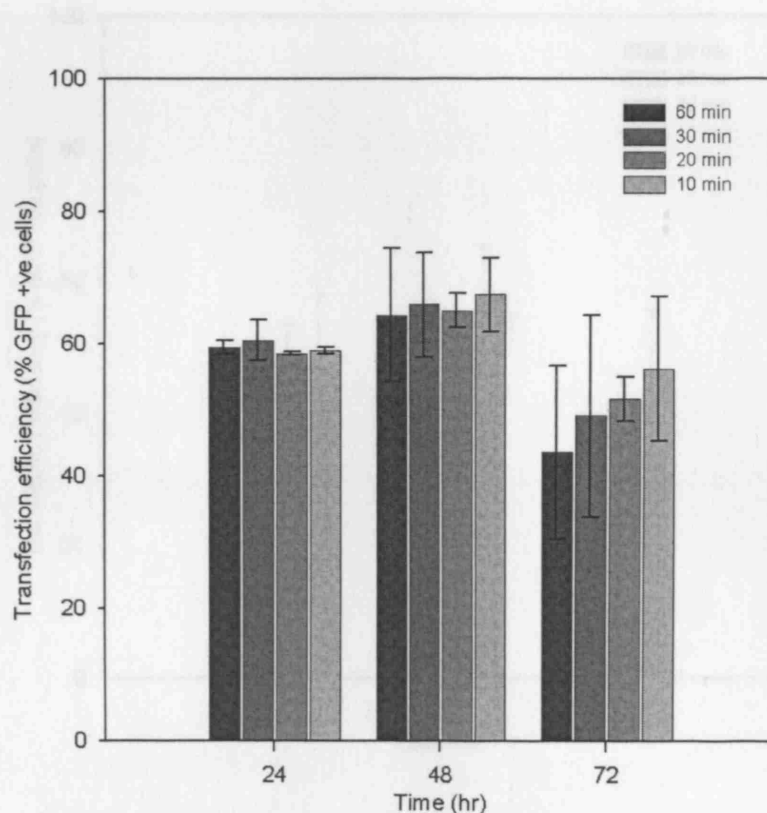


Figure 5.2a Transfection efficiencies of CHO-S cells transfected with PEI-DNA complexes formed in 150 mM NaCl for 10-60 minutes. PEI-DNA complexes were formed in 150mM NaCl physiological buffer for 10, 20 30 and 60 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured on CHO-S-SFM II media in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes added at time 0 hr. The number of GFP expressing cells was determined by flow cytometry as described in Section 3.3.5. Results show typical GFP expression levels from at least two replicate experiments. Error bars represent one standard deviation about the mean.

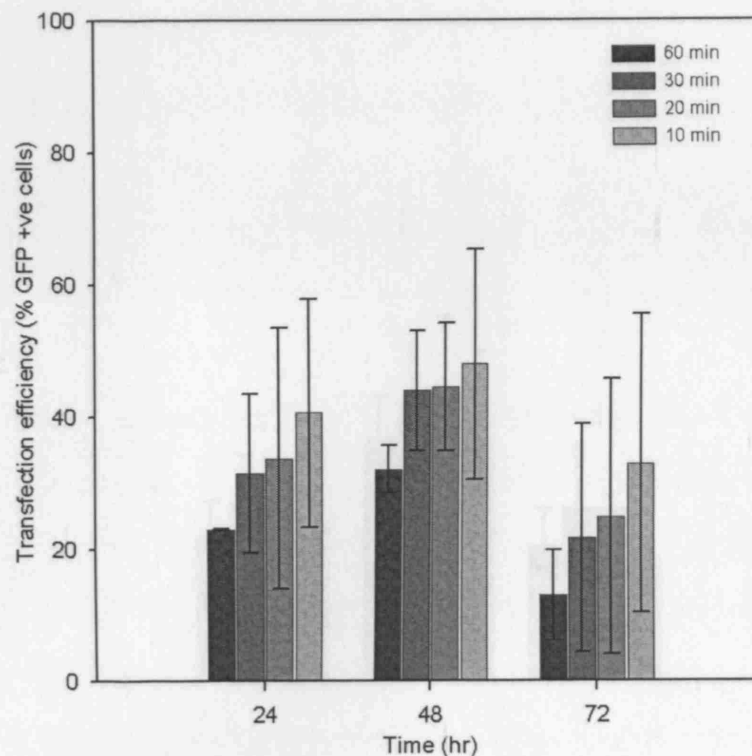


Figure 5.2b Transfection efficiencies of CHO-S cells transfected with PEI-DNA complexes formed in DMEM for 10-60 minutes. PEI-DNA complexes were formed in DMEM basal culture media for 10, 20 30 and 60 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured on CHO-S-SFM II media in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes added at time 0 hr. The number of GFP expressing cells was determined by flow cytometry as described in Section 3.3.5. Results show typical GFP expression levels from at least two replicate experiments. Error bars represent one standard deviation about the mean.

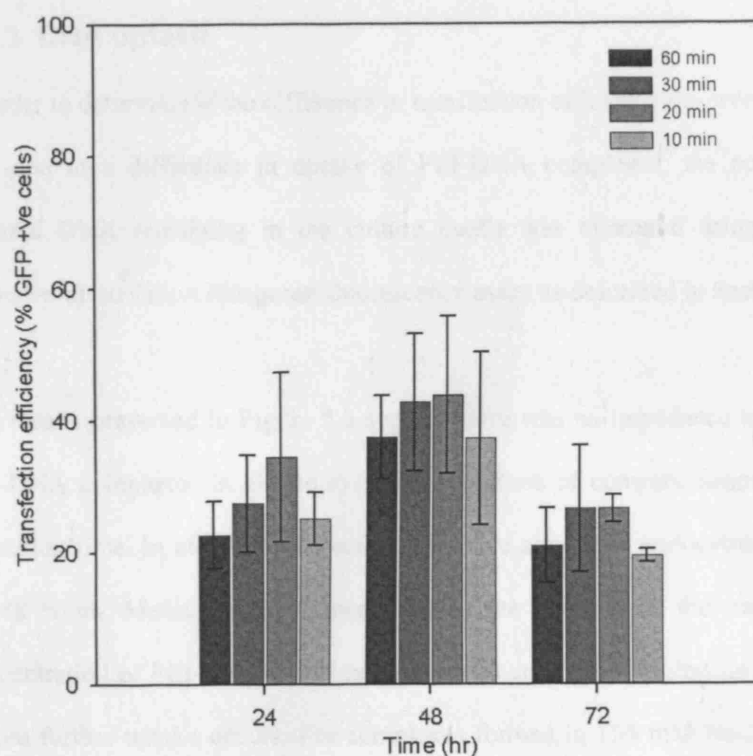


Figure 5.2c Transfection efficiencies of CHO-S cells transfected with PEI-DNA complexes formed in CHO-S-SFM II for 10-60 minutes. PEI-DNA complexes were formed in CHO-S-SFM II culture media for 10, 20 30 and 60 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured on CHO-S-SFM II media in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes added at time 0 hr. The number of GFP expressing cells was determined by flow cytometry as described in Section 3.3.5. Results show typical GFP expression levels from at least two replicate experiments. Error bars represent one standard deviation about the mean.

5.2.3 DNA uptake

In order to determine if the difference in transfection efficiency observed in Figure 5.2 was due to a difference in uptake of PEI-DNA complexes, the concentration of plasmid DNA remaining in the culture media was measured using the Heparin-mediated dissociation Picogreen fluorescence assay as described in Section 3.3.6.

The results presented in Figure 5.3 suggest there was no impedance to the uptake of PEI-DNA complexes in all the systems, regardless of complex formation buffer or formation time. In all cases, the complexes were almost all endocytosed by the cells by 48 hours. Measurements showed that in the majority of the transfections, the concentration of PEI-DNA complexes remained around 500 ng/ml up until 24 hours, before further uptake occurs. For complexes formed in 150 mM NaCl (Figure 5.3a) and CHO-S-SFM II culture media (Figure 5.3c), almost complete uptake was observed by 36 hours. For complexes formed in CHO-S-SFM II, there was no significant difference in the uptake profile of complexes with different incubation times.

A similar plateau was observed from 0 to 24 hours, although the uptake profile for complexes formed in 150mM NaCl has larger differences between those with different incubation times than measured for complexes formed in culture media. The complexes formed for 60 minutes and 30 minutes were more readily taken up than the complexes formed for 20 minutes and 10 minutes. A small rise was measured in the DNA concentration between 6 hours and 12 hours. Complexes formed for 10 minutes

attained more than 90% uptake only after 48 hours; whereas the other complexes formed in 150 mM NaCl were taken up by 36 hours.

The plasmid DNA uptake profile for complexes formed in DMEM (Figure 5.3b) showed some variation between the complexes incubated for different periods of time. Between 0 and 24 hours, the concentration of plasmid DNA in the culture media varied between 250 ng/ml and 650 ng/ml. The largest variation was measured in complexes formed for 20 minutes and 10 minutes. In all transfections with DMEM complexes there was a large drop in plasmid DNA concentration between 24 hours and 48 hours, by which almost all the complexes had been removed from the culture media.

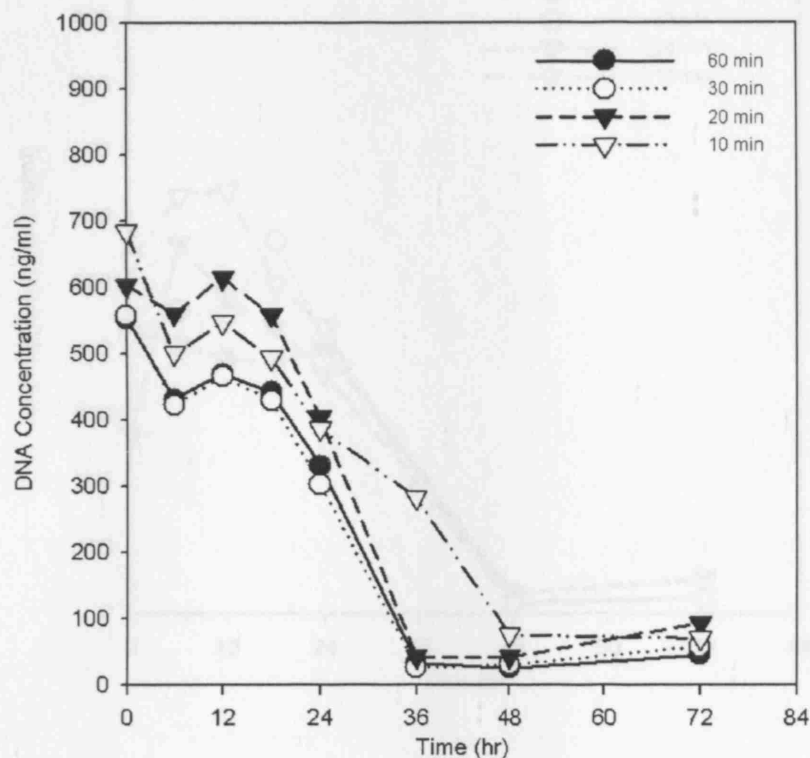


Figure 5.3a Concentration of DNA remaining in the supernatant post-transfection for CHO-S cells transfected with PEI-DNA complexes formed in 150 mM NaCl for 10-60 minutes. PEI-DNA complexes were formed in 150mM NaCl physiological buffer for 10, 20 30 and 60 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured on CHO-S-SFM II media in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes added at time 0 hr. DNA concentration in the culture media was determined using with the Picogreen assay as described in Section 3.3.6. Results show averaged DNA concentrations from at least two replicate experiments.

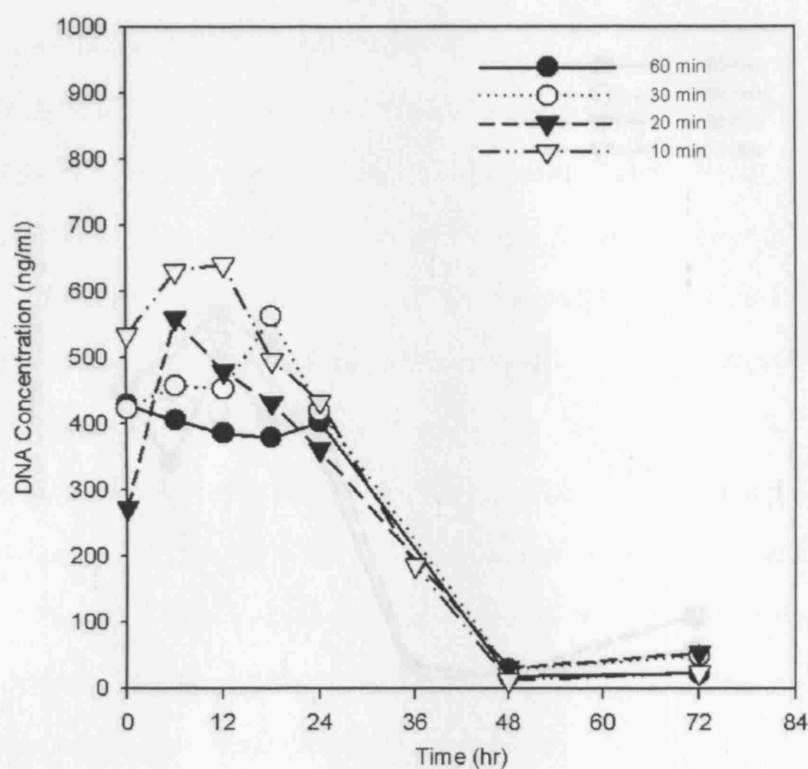


Figure 5.3b Concentration of DNA remaining in the supernatant post-transfection for CHO-S cells transfected with PEI-DNA complexes formed in DMEM for 10-60 minutes. PEI-DNA complexes were formed in DMEM basal media for 10, 20 30 and 60 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured on CHO-S-SFM II media in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes added at time 0 hr. DNA concentration in the culture media was determined using with the Picogreen assay as described in Section 3.3.6. Results show averaged DNA concentrations from at least two replicate experiments.

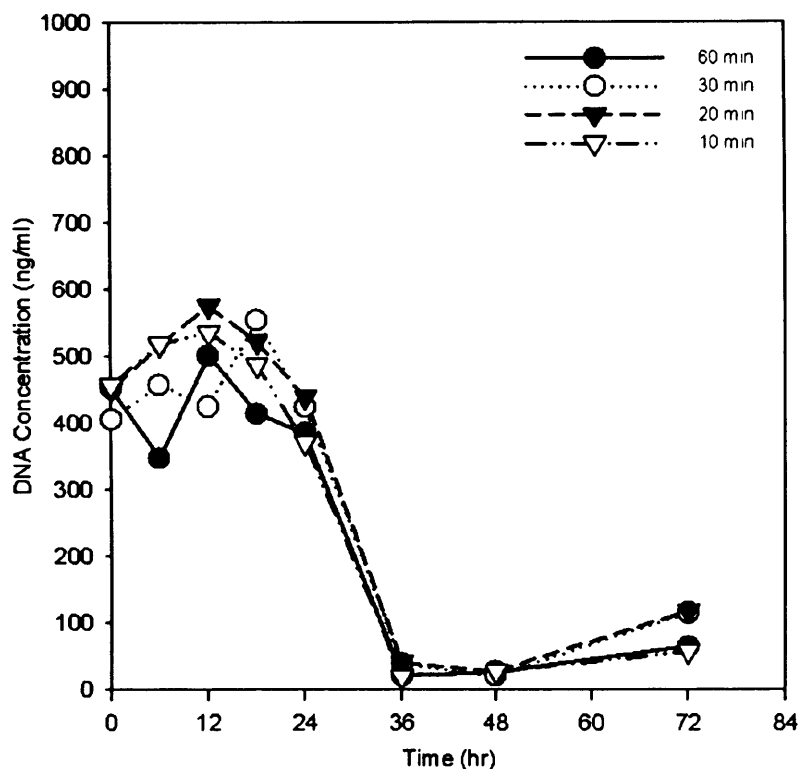


Figure 5.3c Concentration of DNA remaining in the supernatant post-transfection for CHO-S cells transfected with PEI-DNA complexes formed in CHO-S-SFM II for 10-60 minutes. PEI-DNA complexes were formed in CHO-S-SFM II culture media for 10, 20 30 and 60 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured on CHO-S-SFM II media in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes added at time 0 hr. DNA concentration in the culture media was determined using with the Picogreen assay as described in Section 3.3.6. Results show averaged DNA concentrations from at least two replicate experiments.

5.2.4 Particle size measurements

As described in Section 4.2, PEI-DNA complexes aggregate over a formation period of 60 minutes. For complexes formed in 150 mM NaCl for 60 minutes, a gradual decrease in size was observed when diluted in CHO-S-SFM II (Section 4.3). Therefore, for experiments in which the DNA/PEI complexes are incubated for different periods of time, the size of the complexes would conceivably vary in size when transfecting the cells. In order to follow the changes in the particle characteristics following transfection, the particle size and count were measured as described in Section 3.3.3. The initial measurements taken upon transfection showed that there was not a large particle size difference for the complexes upon dilution when added to the cells (Figure 5.4). In all cases, there was a gradual trend of increase in the particle size over 24 hours. The measure complex sizes grew from around 200 nm at 0 hour to around 250 nm at 24 hours. A drop in complex size followed this to around 100 nm at 48 hours.

5.2.5 Particle count measurements

The number of PEI-DNA complexes remaining in the supernatant post transfection was also determined in order to provide additional insight into the behaviour of the complexes post-transfection and also gives an indication of complex uptake.

Generally, a plateau in the particle count was observed between 0-24 hours in all cases. The initial particle counts were higher, from 17.6 KCps (10 minute incubation time) to 20 KCps (20 minute incubation time) for complexes formed in 150mM NaCl (Figure 5.5a). The slow increase in particle count observed in transfections with complexes formed in culture media was not observed with complexes formed in

150mM NaCl. There did not appear to be a trend with the early particle count measurements and the formation incubation time for complexes formed in 150mM NaCl. There was a small rise in the particle count for complexes formed in DMEM over 12 hours (Figure 5.5b). Initial particle count measurements appeared to be in between that observed for those measured in CHO-S-SFM II and 150mM NaCl complexes, from 14 KCps (10 minute incubation) to 17 KCps (20 minute incubation). Once again there did not appear to be a trend in initial particle counts and complex formation incubation time. The particle counts started to decrease after 12 hours, earlier than complexes formed in 150mM NaCl and CHO-S-SFM II, reaching around 1 KCps after 48 hours post transfection. The particle count profile for CHO-S-SFM II (Figure 5.5c) and DMEM (Figure 5.5b) were quite similar. Initial measurements of particle count were around 15 KCps (Figure 5.5c). This slowly increased post-transfection over a period of 24 hours from 11 KCps (60 minute incubation time) – 15.6 KCps (10 minute incubation time) initially to around 18 KCps at 24 hours. The initial particle count decreased as the formation incubation time of the complexes increased. There was a sharp drop in the particle count by 48 hours to about 2 KCps.

Overall, the PEI-DNA complexes formed in 150mM NaCl for 10 minutes gave the highest transfection efficiency. These conditions for complex formation were used in subsequent investigations.

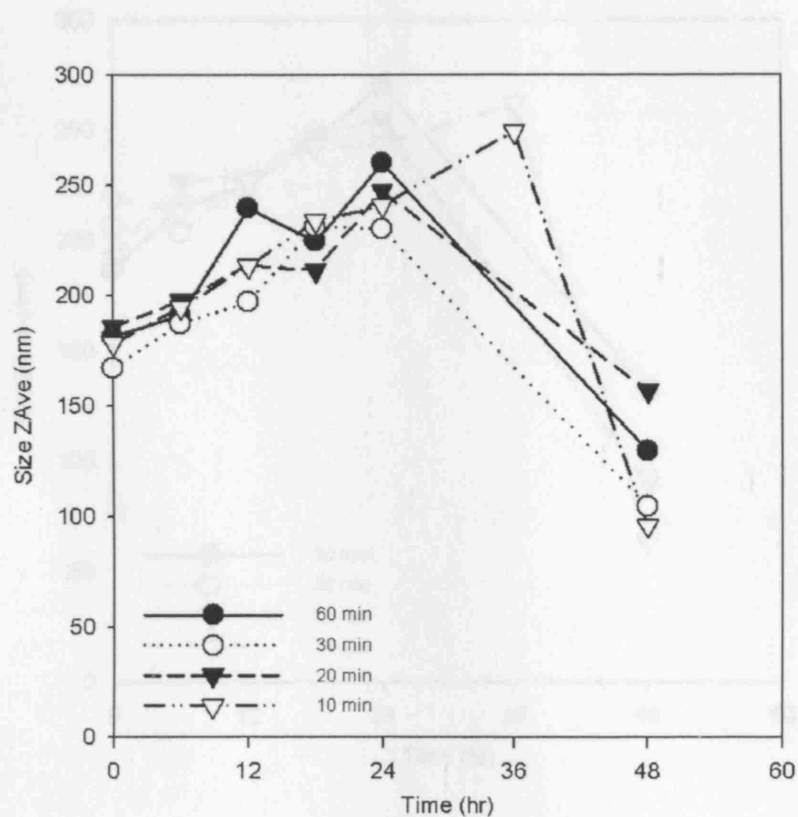


Figure 5.4a Change in supernatant PEI-DNA complex particle size post-transfection of CHO-S with complexes formed in 150 mM NaCl for 10-60 minutes. CHO-S cells transfected, as described in legend to Figure 5.1a, Particle size was measured with a Zetasizer as described in Section 3.3.3. Results show mean particle sizes from at least two replicate experiments.

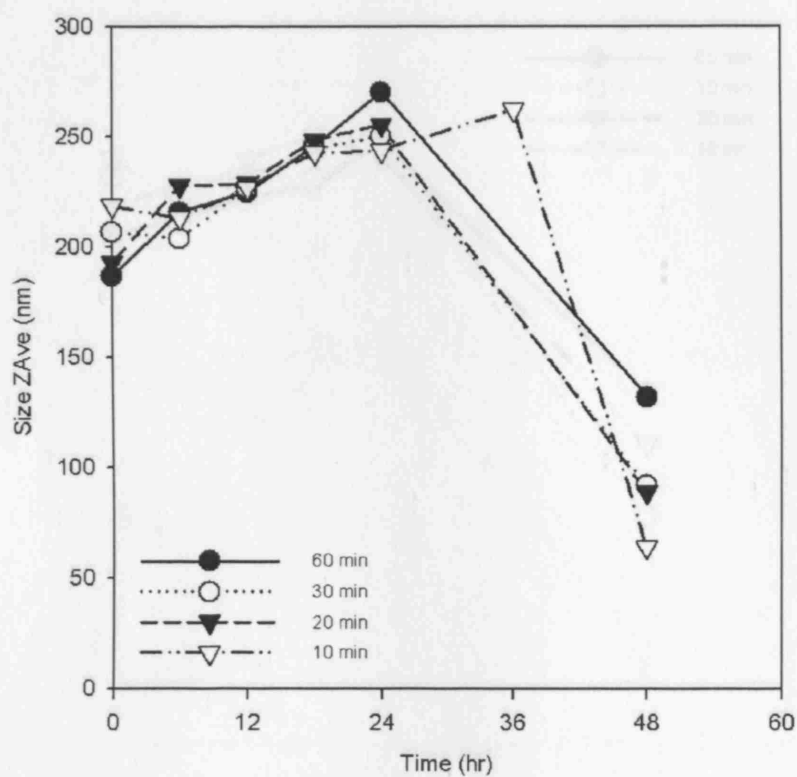


Figure 5.4b Change in supernatant PEI-DNA complex particle size post-transfection of CHO-S with complexes formed in DMEM for 10-60 minutes. CHO-S cells transfected, as described in legend to Figure 5.1b, Particle size was measured with a Zetasizer as described in Section 3.3.3. Results show mean particle sizes from at least two replicate experiments.

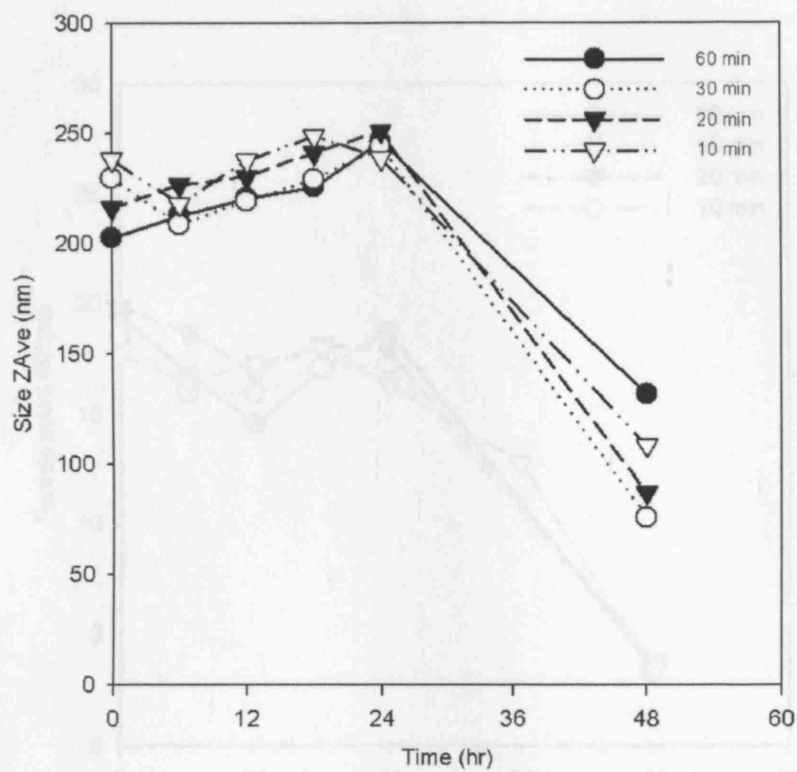


Figure 5.4c Change in supernatant PEI-DNA complex particle size post-transfection of CHO-S with complexes formed in CHO-S-SFM II for 10-60 minutes. CHO-S cells transfected, as described in legend to Figure 5.1c, Particle size was measured with a Zetasizer as described in Section 3.3.3. Results show mean particle sizes from at least two replicate experiments.

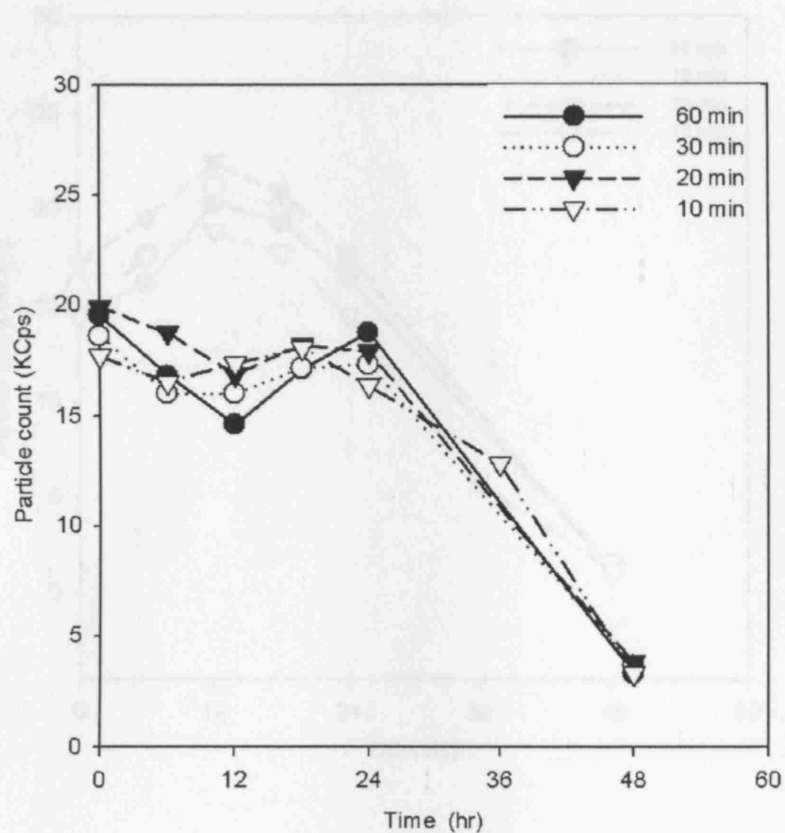


Figure 5.5a Change in supernatant PEI-DNA complex particle count post-transfection of CHO-S with complexes formed in 150 mM NaCl for 10-60 minutes. CHO-S cells transfected, as described in legend to Figure 5.1a, Particle count was measured with a Zetasizer as described in Section 3.3.3. Results show mean particle counts from at least two replicate experiments.

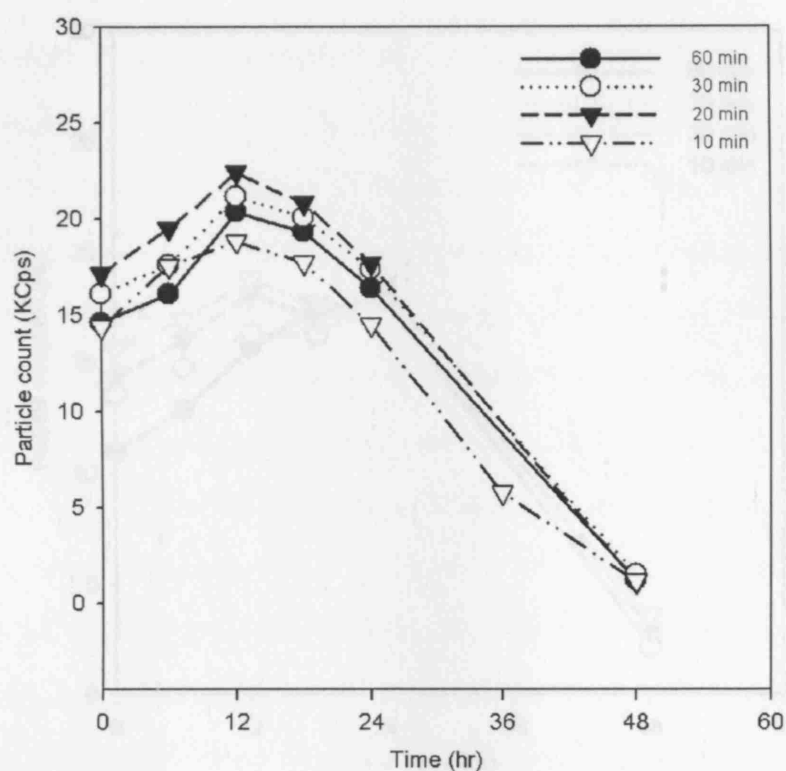


Figure 5.5b Change in supernatant PEI-DNA complex particle count post-transfection of CHO-S with complexes formed in DMEM for 10-60 minutes. CHO-S cells transfected, as described in legend to Figure 5.1b, Particle count was measured with a Zetasizer as described in Section 3.3.3. Results show mean particle counts from at least two replicate experiments.

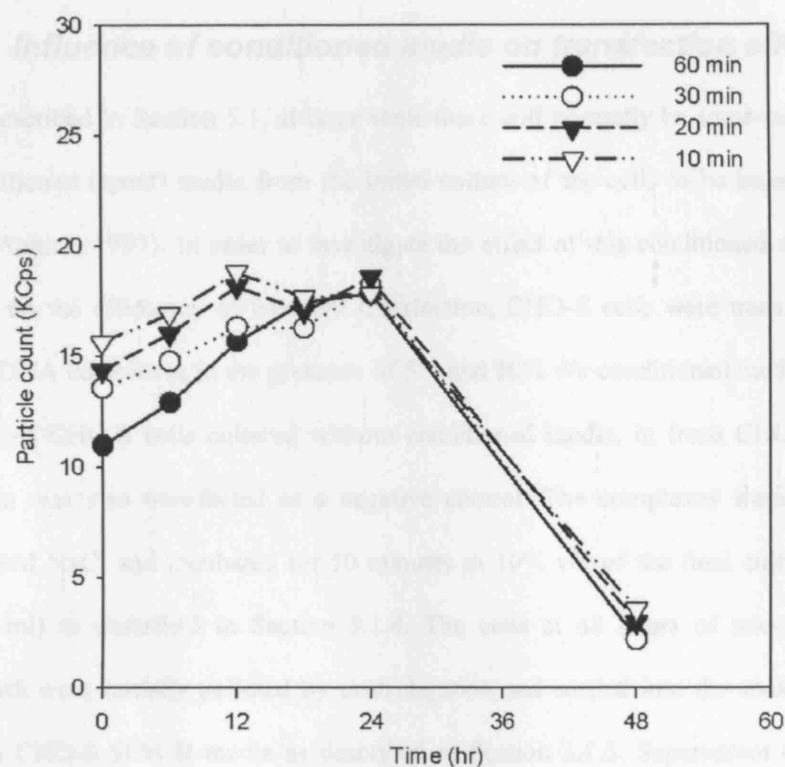


Figure 5.5c Change in supernatant PEI-DNA complex particle count post-transfection of CHO-S with complexes formed in CHO-S-SFM II for 10-60 minutes. CHO-S cells transfected, as described in legend to Figure 5.1c, Particle count was measured with a Zetasizer as described in Section 3.3.3. Results show mean particle counts from at least two replicate experiments.

5.3.1 Cell growth

The cell growth of CHO-S cells transfected with the complexes formed in CHO-S-SFM II was monitored over a period of 48 hours. The cell growth was measured by the optical density (OD) at 550 nm. The results showed that the cell growth was not significantly affected by the transfection process. The cell growth was similar to the control cells (CHO-S cells transfected with CHO-S-SFM II only). The cell growth was measured at 0, 12, 24, and 48 hours. The cell growth was similar to the control cells (CHO-S cells transfected with CHO-S-SFM II only). The cell growth was measured at 0, 12, 24, and 48 hours. The cell growth was similar to the control cells (CHO-S cells transfected with CHO-S-SFM II only).

5.3 Influence of conditioned media on transfection efficiency

As described in Section 5.1, at large scale there will normally be some carry-over of conditioned (spent) media from the initial culture of the cells to be transfected (Hu and Aunins, 1997). In order to investigate the effect of this conditioned media carry over on the efficiency of transient transfection, CHO-S cells were transfected with PEI-DNA complexes in the presence of 5% and 10% v/v conditioned media. A shake flask of CHO-S cells cultured without conditioned media, in fresh CHO-S-SFM II media was also transfected as a negative control. The complexes were formed in 150mM NaCl and incubated for 10 minutes in 10% v/v of the final culture volume (2.5 ml) as described in Section 3.1.4. The cells at 48 hours of mid-exponential growth were initially pelleted by centrifugation and seeded into the shake flasks of fresh CHO-S-SFM II media as described in Section 3.1.5. Supernatant conditioned media from the centrifugation step was added to the culture at 5 % and 10% v/v of the 25 ml final culture volume. The cells were subsequently transfected with complexes as described in Section 3.1.5. The seeding viable CHO-S cell concentration for transfection was 2.0×10^5 cells/ml.

5.3.1 Cell growth

The cell density at the end of the culture increased with an increasing percentage of conditioned media present in the transfection (Figure 5.6). The highest final cell density of 3.4×10^6 cells/ml was measured at 96 hours in the transfection performed with 10% v/v conditioned media present. The transfection with no conditioned media achieved a final cell density of 2.4×10^6 cells/ml.

5.3.2 Transfection efficiency

As shown in Figure 5.7, increasing the volume fraction of conditioned media in the culture media decreases the transfection efficiency of PEI-DNA complexes formed in 150mM NaCl. The negative control culture transfected in fresh media, without the addition of 48 hour conditioned media, gave the highest transfection efficiency at 48 hours of 63 % GFP positive cells (Figure 5.7). The transfection with 10% v/v conditioned media present gave the lowest transfection efficiency at 48 hours, with 40.3% GFP positive cells. In all cases, the highest transfection efficiency was measured at 48 hours, followed by a decrease in GFP positive cells at 72 hours.

5.3.3 DNA uptake

In order to determine whether the difference observed in the transfection efficiencies of the cultures with increasing amount of conditioned media present was due to complex uptake by the cells, the uptake of plasmid DNA was measured using the heparin mediated dissociation Picogreen assay (Section 3.2.4). As shown in Figure 5.8 the initially measured DNA concentrations for transfections with 5 and 10% v/v conditioned media were much higher than those for the transfection in fresh media. The DNA concentration measured at 0 hr post-transfection in the presence of conditioned media was approximately 1.5-fold higher than for the non-conditioned media experiment. In all three cases DNA was readily taken up into the CHO-S cells; by 48 hours almost all the complexes had been endocytosed.

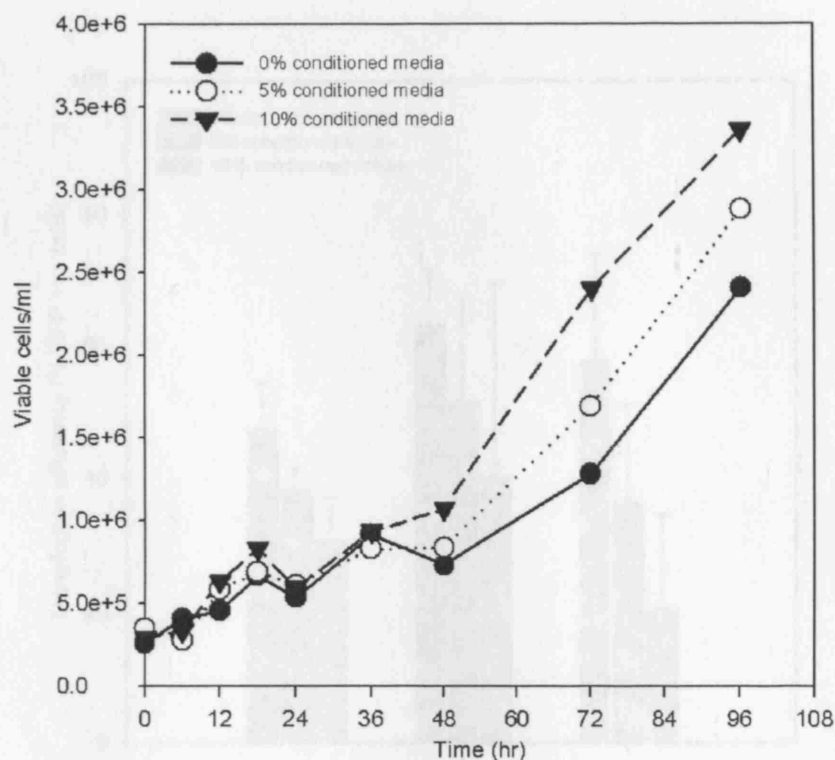


Figure 5.6. Growth kinetics of transfected CHO-S cells cultured in CHO-S-SFM II culture media supplemented with 10%, 5% and 0% v/v conditioned media. Cells were transfected with PEI-DNA complexes formed in 150mM NaCl physiological buffer for 10 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes were added at time 0 hr. Viable cell counts performed as described in Section 3.3.2. Results show typical profiles from at least two replicate experiments.

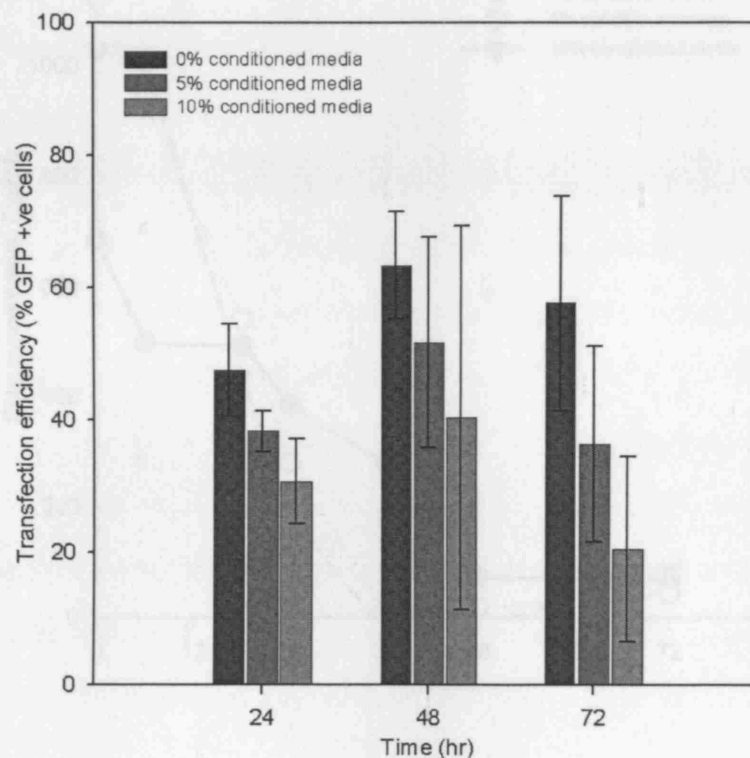


Figure 5.7. Transfection efficiencies of transfected CHO-S cells cultured in CHO-S-SFM II culture media supplemented with 10%, 5% and 0% v/v conditioned media. Cells were transfected with PEI-DNA complexes formed in 150mM NaCl physiological buffer for 10 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes were added at time 0 hr. The number of GFP expressing cells was determined by flow cytometry as described in Section 3.3.5. Results show typical GFP expression levels from at least two replicate experiments. Error bars represent one standard deviation about the mean.

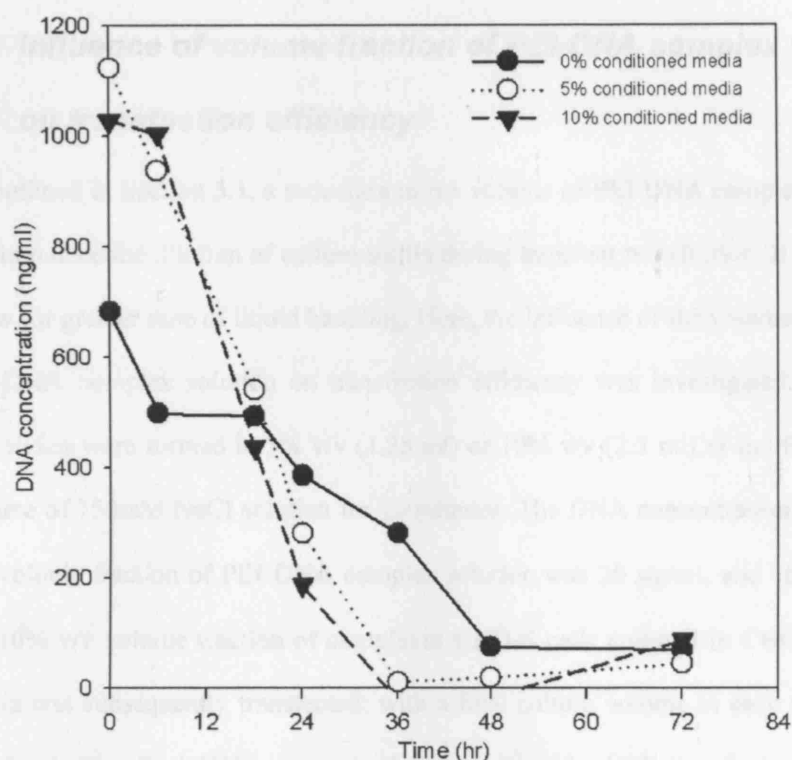


Figure 5.8 Concentration of DNA remaining in the supernatant post-transfection of transfected CHO-S cells cultured in CHO-S-SFM II culture media supplemented with 10%, 5% and 0% v/v conditioned media. Cells were transfected with PEI-DNA complexes formed in 150mM NaCl physiological buffer for 10 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes were added at time 0 hr. DNA concentration in the culture media was determined using with the Picogreen assay as described in Section 3.3.6. Results show averaged DNA concentrations from at least two replicate experiments.

5.4 Influence of volume fraction of PEI-DNA complex solution on transfection efficiency

As outlined in Section 5.1, a reduction in the volume of PEI-DNA complex solutions would reduce the dilution of culture media during transient transfection. It would also allow for greater ease of liquid handling. Here, the influence of the volume fraction of PEI-DNA complex solution on transfection efficiency was investigated. PEI-DNA complexes were formed in 5% v/v (1.25 ml) or 10% v/v (2.5 ml) of the final culture volume of 150mM NaCl solution for 10 minutes. The DNA concentration for the 5% v/v volume fraction of PEI-DNA complex solution was 20 µg/ml, and 10 µg/ml for the 10% v/v volume fraction of complexes CHO-S cells cultured in CHO-S-SFM II media was subsequently transfected; with a final culture volume in each shake flask of 25 ml. The final DNA concentration in culture for both transfections was 1.0 µg/ml.

5.4.1 Cell growth

As shown in Figure 5.9, transfections with complexes formed in 5% v/v of the final culture volume achieved much higher cell densities at 96 hours than transfections with complexes formed in 10% v/v volume fraction. Both transfections had cell densities which were fairly similar up to 24 hours post-transfection. Only after 24 hours, was there a divergence in the cell concentration between the two transfection procedures. The transfection with 5% v/v complexes reached a final cell density of 3.14×10^6 cells/ml at 96 hours in contrast to the transfection with 10% v/v complexes, which only reached 1.70×10^6 cells/ml. Cell viability however remained high, > 90% viable cells, in both cases.

5.4.2 Transfection efficiency

As shown in Figure 5.10, the percentage of GFP expressing cells was slightly higher for the transfection with 10% v/v complexes than the transfection with 5% v/v complexes (Figure 5.10). The peak transfection efficiency was measured at 48 hours for both transfections. Cells transfected with 10% v/v complexes achieved a transfection efficiency of 62.7% GFP expressing cells, as compared with 53.0% for the transfection with 5% v/v complexes.

5.4.3 DNA uptake

PEI-DNA complexes were readily taken up in both transfections with 5% and 10% v/v complexes (Figure 5.11). There was a rapid decrease in DNA concentration present in the supernatant between 12 and 24 hours in both transfections. In both transfections, the DNA concentration remaining in the supernatant decreased by more than 90%, from the initial 1000 ng/ml added, to less than 100 ng/ml by 48 hours.

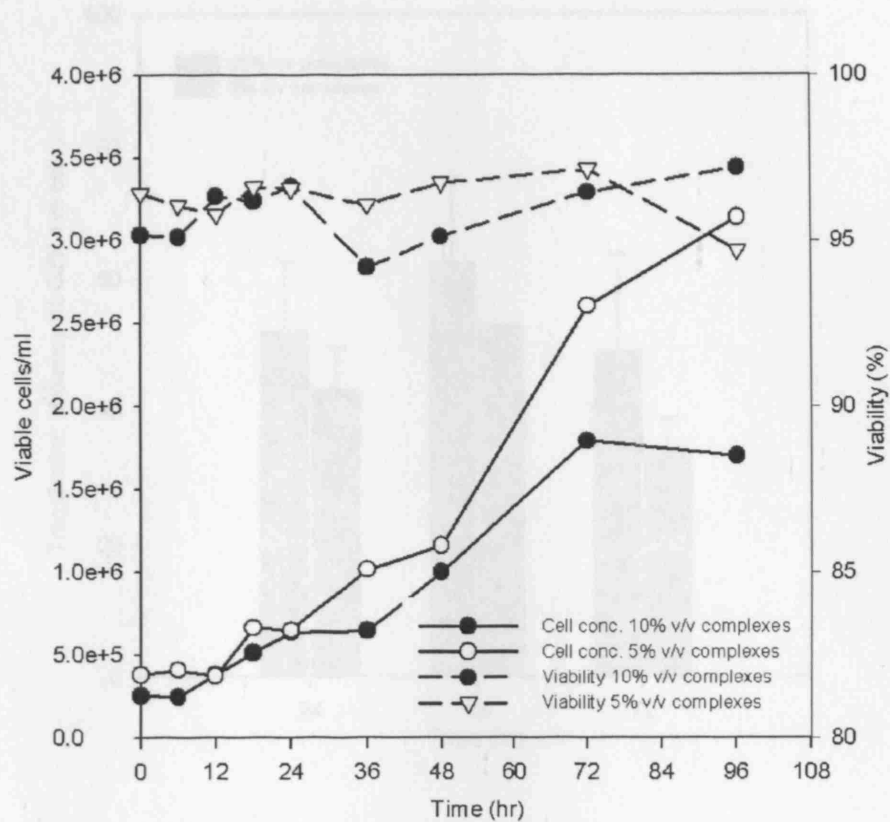


Figure 5.9 Growth kinetics and viability of CHO-S cells transfected with 5 and 10% volume fraction of PEI-DNA complex solution. Cells were transfected with PEI-DNA complexes formed in 150mM NaCl physiological buffer for 10 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes were added at time 0 hr. Viable cell counts performed as described in Section 3.3.2. Results show typical profiles from at least two replicate experiments.

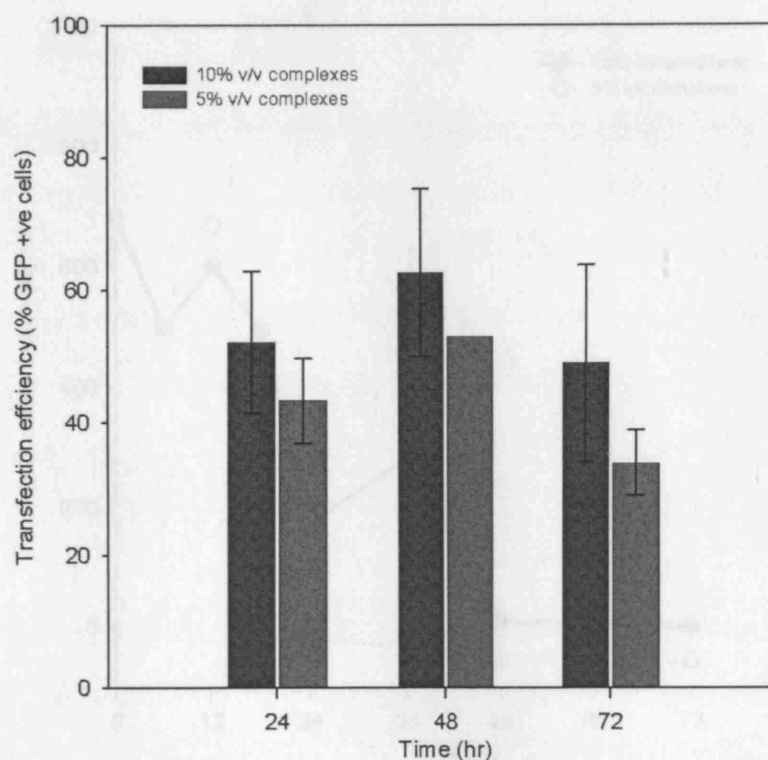


Figure 5.10 Transfection efficiency of CHO-S cells transfected with 5 and 10% volume fraction of PEI-DNA complex solution. Cells were transfected with PEI-DNA complexes formed in 150mM NaCl physiological buffer for 10 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes were added at time 0 hr. The number of GFP expressing cells was determined by flow cytometry as described in Section 3.3.5. Results show typical GFP expression levels from at least two replicate experiments. Error bars represent one standard deviation about the mean.

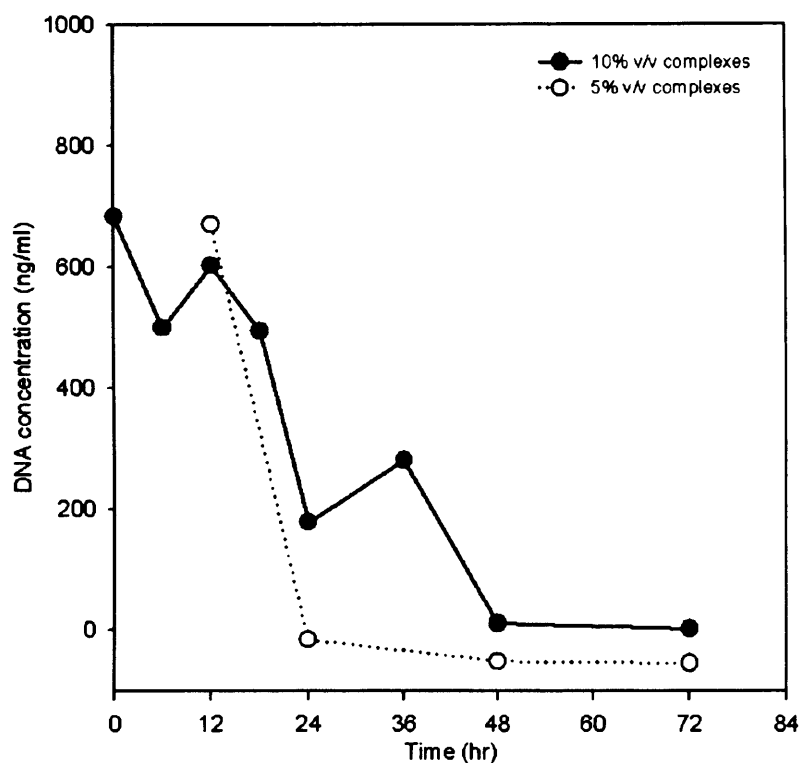


Figure 5.11 Concentration of DNA remaining in the supernatant post-transfection of CHO-S cells transfected with 5 and 10% volume fraction of PEI-DNA complex solution. Cells were transfected with PEI-DNA complexes formed in 150mM NaCl physiological buffer for 10 minutes under static conditions, at room temperature as described in Section 3.1.4. CHO-S cells were cultured in 125 ml shake flasks as described in Section 3.1.5. PEI-DNA complexes were added at time 0 hr. DNA concentration in the culture media was determined using with the Picogreen assay as described in Section 3.3.6. Results show averaged DNA concentrations from at least two replicate experiments.

5.5 Discussion

5.5.1 Effect of complex formation time on transfections

When a process is scaled up to larger volumes, the tight control of parameters becomes more difficult to achieve. When dealing with larger volumes of PEI-DNA complex transfection solutions during scaling up, factors such as mixing and pumping time become increasingly significant. As such, short complex formation times of 5-10 minutes become difficult to control. Thus, in Section 5.2 the effect longer complex formation times have on transfections with PEI-DNA complexes was investigated.

For cells transfected with PEI-DNA complexes formed in 150mM NaCl, there was no significant variation in the peak transfection efficiency at 48 hours between the complexes with formation incubation times of 10-60 minutes (Figure 5.2a). This is similar to the findings previously reported on complexes formed in 150 mM NaCl (Derouazi et al, 2004), where the incubation time was investigated up to 20 minutes. Complexes formed for 10 minutes gave somewhat higher transfection efficiency of 67.5% GFP expressing cells at 72 hours. By 96 hours the correlation between the transfection efficiency and cell growth became more apparent: As observed before, lower transfection efficiency lead to higher cell densities at 96 hours (Figure 5.1a).

There were differences observed in the plasmid DNA uptake up to 36 hours (Figure 5.3a). Complexes formed in 150mM NaCl appeared to be more readily taken up than complexes formed in culture media. Slightly faster uptake was measured for complexes formed for 60, 30 and 20 minutes, plasmid DNA was almost all taken up

by 36 hours. Initial differences could be due to the varying size of particles during transfection from the longer formation times.

Aggregation of complexes formed in 150mM NaCl during the formation stage, reported in Section 4.2, would most likely lead to differently sized complexes during the transfection. Initial particle size measurements after transfection, to determine whether this was the case, showed that the complexes rapidly adjusted to the new buffer environment. The particle sizes measured immediately after transfection were very similar, but there was a difference in the initial particle counts (Figure 5.4a). As the complexes aggregated over time, the particle counts would subsequently decrease as well. Aggregated complexes, which have longer incubation times, would present lower early particle counts upon transfection than complexes incubated for a shorter period of time. The trend of increase in the particle size up to 24 hours with a corresponding slight decrease in the particle counts between 0 to 24 hours (Figure 5.5a) is most probably due to further aggregation of the complexes in the culture. The aggregation is mostly due to the plateau in the uptake between 0-24 hours (Figure 5.3a). Aggregation post-transfection would most likely also incorporate the biological material in the culture media, including waste products from cells etc. This would show up as an increase in the particle size without a corresponding drop in the particle count.

Transfection efficiency for PEI-DNA complexes formed in DMEM appears to be influenced by the complex formation incubation time: increasing the incubation time lead to a decrease in the transfection efficiency at 48 hours (Figure 5.2b). This is similar to the results from transfections with complexes formed in CHO-S-SFM II

(Figure 5.2c). This is most probably due to the similarities in chemical composition between CHO-S-SFM II and DMEM. The increase in particle size of the complexes between 0 and 24 hours did not have a corresponding decrease in particle size. The variation in the DNA concentrations between 0 hour and 24 hours (Figure 5.3b) are most likely due to interference from cell lysis, or errors in the sampling technique. Once again, the complexes were readily taken up into the cells by 48 hours.

The cell growth profiles of CHO-S cells transfected with PEI-DNA complexes formed in CHO-S-SFM II follow the trend previously described in Section 4.5.1. Higher transfection efficiency lead to lower peak cell density at 96 hours (Figure 5.1c). Complexes incubated for 10 minutes did not give the highest transfection efficiency, as expected (Figure 5.2c). The transfection efficiency increased with decreasing formation time for CHO-S-SFM II complexes, until 10 minutes. The DNA uptake profiles show that complexes were readily taken up, despite the increasing formation times (Figure 5.3c). There also did not appear to be a significant difference in the particle size (Figure 5.4c) or particle count (Figure 5.5c) for the complexes after transfection. From the studies carried out on the formation of PEI-DNA complexes in CHO-S-SFM II (Section 4.3), the similar buffer environments lead to rapid adjustment of the complexes upon transfection. This would coincide with the similar uptake profiles. There is a critical point where the aggregations of the complexes encourage DNA release at 20 minutes. This could be due to the disruptive effects of PEI (Ruponen et al, 2003; Sonawane et al, 2003b) or the topology of the complexes (Tang and Szoka, 1997). At this point the reason for decreased transfection efficiency with increasing complex formation incubation time, for complexes formed in media, is unclear. Future work to be undertaken as described in Section 7.5.2.

In general, it was found that transfection efficiencies of PEI-DNA complexes prepared in 150 mM NaCl physiological buffer were much less affected by formation incubation time than complexes prepared in culture media. As suggested in Section 4.6.1, the similar composition of CHO-S-SFM II and DMEM is probably responsible for the similar trends in transfection efficiency. Process scale application would require robustness, making 150 mM NaCl, or a similar aqueous solution, a suitable choice for PEI-DNA complex formation.

In summary, measurements of the particle sizes of PEI-DNA complexes formed in different aqueous environments show that the complexes rapidly equalize to between 170-240 nm in size upon transfection (Figure 5.4). This is similar to findings previously reported where complexes formed in 150 mM and 5% glucose also rapidly equalized in size once in contact with culture medium (Breunig et al, 2007).

From this series of investigations into the formation conditions PEI-DNA complexes, it was determined that complexes formed in 150 mM NaCl, then incubated for 10 minutes, gave the highest transfection efficiency. Complexes were thus formed under these conditions for the subsequent experiments.

5.5.2 Effect of conditioned media on PEI-DNA transfection

The specific effect of the presence of conditioned media on transient transfections of CHO-S cells with PEI-DNA complexes has not previously been investigated. From the experimental data presented in Section 5.3, there is a clear correlation between the presence of conditioned media in the culture, and the transfection efficiency of PEI-

DNA complexes. Increased presence of conditioned media from carry-over during expansion leads to lower transfection efficiencies (Figure 5.7). The presence of conditioned media did not appear to affect the uptake of plasmid DNA. In the three concentrations of conditioned media investigated, all DNA was taken up by 48 hours (Figure 5.8). Initial high concentrations of DNA measured could be due to the contamination of genomic DNA carried over from the previous subculture.

There was quite large variability in the transfection efficiency when 10% v/v conditioned media was present. The variability in the conditioned media supplemented into the culture was most likely to be responsible for this. As the complexes are formed at an N/P ratio of 10, there is quite a significant excess of PEI in the transfection mix (Clamme et al, 2003a). The presence of genomic DNA (gDNA) (from lysed cells) carried over in the conditioned media from the previous culture might form new PEI-gDNA complexes with excess PEI present. These complexes would then mostly likely compete with the PEI-plasmid DNA complexes for uptake sites on the CHO-S cell surface (Rejman et al, 2005), thus affecting the uptake of complexes with the transgene.

In order to have tighter process control and minimise the detrimental effects of conditioned media on transfection efficiency, the amount of conditioned media carried over into the transfection during scale-up should be minimized as far as possible.

5.5.3 Effect of volume fraction of PEI-DNA complex solution on transfection efficiency

PEI-DNA complexes are typically formed in either 10% or 5% v/v of the final culture volume (Schlaeger and Christensen, 1999; Derouazi et al, 2004). Decreasing the volume in which the PEI-DNA complexes were formed, while keeping the overall DNA concentration during transfection constant (1 $\mu\text{g/ml}$), did not increase the transfection efficiency (Figure 5.10). It did, however, increase the final cell concentration at 96 hours in the transfection with complexes in 5% volume fraction (Figure 5.9). This might be because of the proportionally greater amount of nutrients available for biomass growth. The higher cell concentration observed in transfections with 5% v/v PEI-DNA complexes could also be linked to the lower transfection efficiency. If the expression of GFP were lower, this would lead to more nutrients available for cell growth, as described in Section 4.6. Another possible reason for lower transfection efficiency in cultures with 5% volume fraction could be the distribution of complexes during transfection. The smaller addition volume might lead to higher localized concentrations of PEI-DNA complexes as previously suggested in Section 5.1, causing the complexes to be unequally distributed throughout the cells. High viabilities in both transfections (Figure 5.9) suggest that the cells were not subject to cytotoxic conditions from the presence of PEI as suggested (Fischer et al, 2003). This could either be because the PEI-DNA complexes were rapidly mixed, without localized high concentrations of PEI, or because the PEI concentrations were not sufficient to induce cytotoxicity.

The findings of reduced transfection efficiency when the formation volume was reduced is the opposite of what had previously been reported for transfections with

Neuro2A cells and PEI-DNA complexes formed in RPMI medium (Ogris et al, 1998). The reason for the decrease in transfection efficiency with a more concentrated PEI-DNA complex suspension is unclear, and requires further investigation.

5.6 Summary

As described in Section 5.1.1, the overall aim of this chapter was to investigate process variables involved in transient transfection, and to determine their effect on transfection efficiency. Process parameters that were investigated were the incubation time of complexes during formation, presence of conditioned media during transfection and the volume fraction of PEI-DNA complexes.

Results in Section 5.2 showed that transfection efficiency of PEI-DNA complexes formed in the simple physiological buffer 150 mM NaCl were less sensitive to changes in the formation incubation time than complexes formed in culture media. In addition, complexes formed in 150 mM NaCl also gave the highest transfection efficiency. Increasing the complex formation time in DMEM and CHO-S-SFM II almost always lead to decreased transfection efficiency in CHO-S cells.

The presence of conditioned media from spent cultures in transfections with PEI-DNA complexes was detrimental to the transfection efficiency. As shown in Section 5.3, increasing the amount of conditioned media lead to a clear decrease in transfection efficiency, and also lead to more variability in the process.

Preparing the PEI-DNA complexes in a smaller volume of formation buffer decreased transfection efficiency. In order to reduce the amount of nutrient dilution during the

transfection process, complexes were formed in 5% v/v of the final culture volume as compared with 10%. Instead, this led to a decrease in transfection efficiency.

From the experiments with different formation media, and complex formation times, PEI-DNA complexes formed in 150mM NaCl solution for 10 minutes gave the best transfection efficiency. The formation buffer for complexes strongly influences the transfection efficiency. During scouting or determination of the best buffer for transfection, if complexes are to be formed in culture media or in the presence of zwitterionic components such as amino acids, the complex formation time should be kept to 10 minutes or less.

Chapter 6

6 Scale up of transient transfection processes: microwell plate to bioreactor

6.1 Introduction

Microwell plates have commonly been used for high-throughput screening and microbial fermentations (Doig et al, 2005). This platform is also useful for cell culture as it allows several process to be investigated (Micheletti and Lye, 2006; Micheletti et al, 2006). 12- and 24-well plates have been used for static culture of adherent cell lines (Ogris et al, 1998; Reed et al, 2006). Recently, there has been a move to use agitated microwell plates for the culture of mammalian cells, and also for transient transfections (Derouazi et al, 2004; Tait et al, 2004).

For the microwell platform to be used for initial process development work, such as improvement of throughput, it must first be demonstrated to be an accurate representation of bioreactor conditions. For these investigations, 24-well Ultra-low attachment (ULA) plates (Corning Life Sciences, Netherlands) were chosen as the platform for agitated microwell plate transient transfections. In order to determine the key parameters involved in scaling the transient transfection process, mixing in the microwell plates and bioreactors was first investigated. The mixing time will give an indication of how quickly the complexes are evenly distributed among the cells. The decolourisation of iodine solution by sodium thiosulphate has been commonly used for mixing time characterization work (Bujalski et al, 1999; Paglianti and Pintus,

2001; Hirata et al, 2007). As the reaction between iodine with sodium thiosulphate is not rate-limiting, the rate-limiting step is liquid phase mixing.

The transfection of PEI-DNA complexes in CHO-S cells has been studied thus far by determining the percentage of cells expressing GFP reporter protein (Chapter 4 and 5). The majority of therapeutic proteins likely to be produced using transient transfection are secreted proteins. In order to correlate transfection efficiency in terms of protein production, Secreted Embryonic Alkaline Phosphatase (SEAP) was selected as the model secreted protein. Transfections with complexes of plasmid DNA encoding for SEAP were carried out on the three different platforms, scaling-up from 1 ml in the 24-well ultra-low attachment plates, to 25 ml in the Erlenmyer shake flask, and finally a 3 l stirred tank bioreactor.

6.1.1 Aim and objectives

This work aims to investigate the application of agitated microwell plates to the transient transfection of mammalian cells and also to demonstrate the scalability of transient transfection. Initially the applicability of microwells for the rapid development of the transient transfection procedure was studied. Followed by scale-up into 3 l bioreactor. The specific objectives of this chapter are to:

- Establish methods for examination of mixing in microwells and bioreactors using high-speed video technology.
- Determine the mixing time and mixing patterns for additions to microwells and bioreactors for different shaking and impeller speeds. Following the high-

speed camera work, the still images were used to elucidate the mixing patterns when carrying out additions to agitated microwell cultures.

- Study the transfection efficiency at different scales in microwell plates and bioreactors under different process conditions.
- Investigate the scalability of transient transfection by determining the recombinant protein productivity across different scales, from microwell plates, to shake flasks and finally in bioreactors. Transient transfections performed in the microwell format were compared to transfection in a 3 l bioreactor.

6.2 Liquid phase mixing in microwell plates

6.2.1 Mixing time measurements for shaken 24-well ultra-low attachment plates.

As outlined in Section 6.1, in order for microwells to be applied to cell culture process development, it must be shown to be an accurate representation of bioreactor processes. For application of microwell plates to transient transfection, the mixing time of PEI-DNA complex solution additions could be a consideration. The initial work described here investigates the mixing time of additions to 24-well ultra-low attachment plates.

Mixing times for 10% v/v and 1% v/v additions in 24-well ultra-low attachment plates were studied. The recommended working fill volume per well was 1 ml. Additions were carried out on the rig described in Section 3.2.1. In order to eliminate the number of variables in the system, additions were performed whilst the microwell mimic was shaking. This was in order to negate the effects of acceleration on the

mixing time. Flexible PEEK tubing (Section 3.2.1) between the fixed pipette for injection and the cap of the microwell allowed this to be performed. The mixing time was defined as the interval between the point where all the sodium thiosulphate had been injected into the iodine, and the point when the solution turns completely colourless. This was determined as described in Section 3.2.2.

From Figure 6.1 it can be seen that the mixing time for additions decreased with increasing shaking speed. Mixing times for conditions below 250 rpm were measured in minutes; mixing time for 10% v/v additions to static plates was 80 minutes. The mixing time at 200 rpm was 11 minutes. It can be seen that there is a clear inflection point in the mixing time between shaking at 200 rpm and 250rpm. Mixing time for additions to the well at 250 rpm were two orders of magnitude faster than the mixing time for additions at 200 rpm. In general, there was little difference between the mixing times for 10% vv additions and 1% v/v additions.

Examining the still images of the additions for 10% v/v additions of thiosulphate (Figure 6.2), an increase in deformation of the liquid surface can be seen with an increase in shaking speed. For additions into static plates, the mixing is mainly observed to be from diffusion. There was an initial amount of jet mixing from the injection of the sodium thiosulphate, but later on, mixing was almost entirely due to diffusion(Nealon et al, 2006). The bottom part of the well, where the thiosulphate is injected, rapidly turns colourless. The liquid surface remains dark brown. Over time, the liquid slowly turns colourless from the bottom up. From 120 to 200 rpm, there is slight deformation on the liquid surface. Decolourisation also occurs upwards from the bottom of the well. As the shaking speed increase to 160 rpm and 200 rpm, from

images taken at 6 seconds, it can be seen that the delineation between the colourless bottom of the well and dark brown surface of the liquid is less defined. The area just beneath the liquid surface is dark yellow, and gradually turns colourless, going from top to bottom. The images taken at 6 seconds for 200 rpm and 225 rpm show a region below the liquid surface that remains brown. The brown region lies just underneath the deformation in the liquid surface. At 225 rpm the liquid surface starts to break up quite significantly. The deformation in the liquid surface becomes deeper and more pronounced. Liquid at the walls of the well have turned colourless and there is a thin clear region between the liquid surface and the brown region that was not observed at 200rpm. When the shaking speed increases to 250 rpm then 300 rpm, the decolourisation occurs very rapidly, under 10 seconds. The deformation in the liquid surface is very pronounced; at 300 rpm it almost touches the bottom of the well. The brown region observed at 120 to 225 rpm is no longer present.

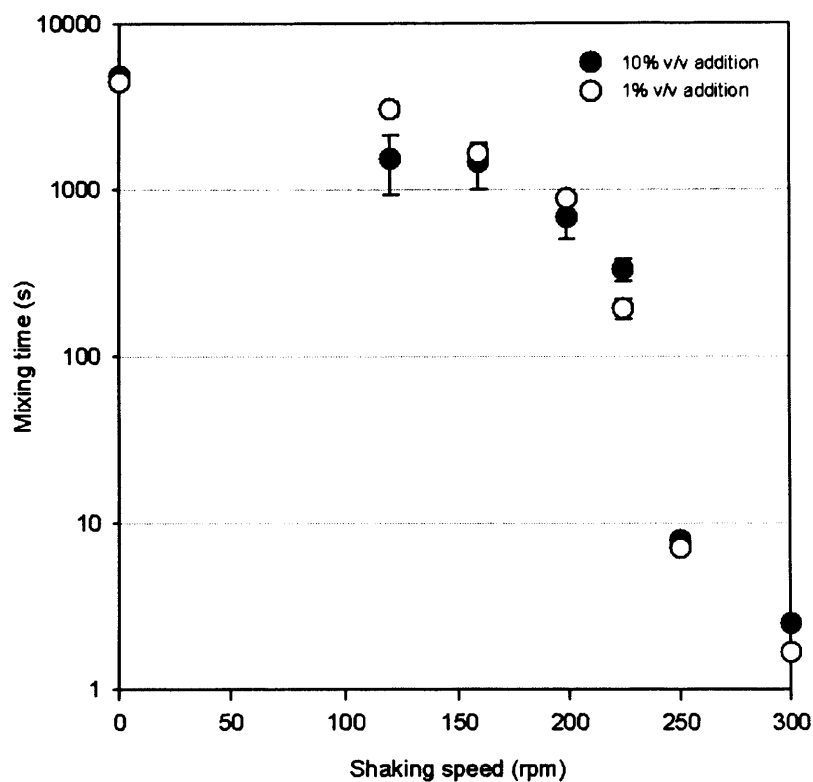


Figure 6.1. Liquid phase mixing time variation with increasing shaking speed measured in 24-well ULA plate for 10% and 1% v/v liquid additions. A 1 ml fill volume was used. For 10% v/v additions, 100 μ l of 90 mM sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) solution was injected into 900 μ l 5mM Iodine (I_2) solution in an agitated well. For 1% v/v additions, 10 μ l of 0.99 M sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) solution was injected into 990 μ l 5mM Iodine (I_2) solution in the well. Additions were performed with the 24-well ULA plate Perspex mimic using the mixing time addition rig described in Section 3.2.1. The microwell mimic was agitated on a Heidolph orbital shaker platform between 0-300 rpm. Error bars represent one standard deviation about the mean of multiple measurements.

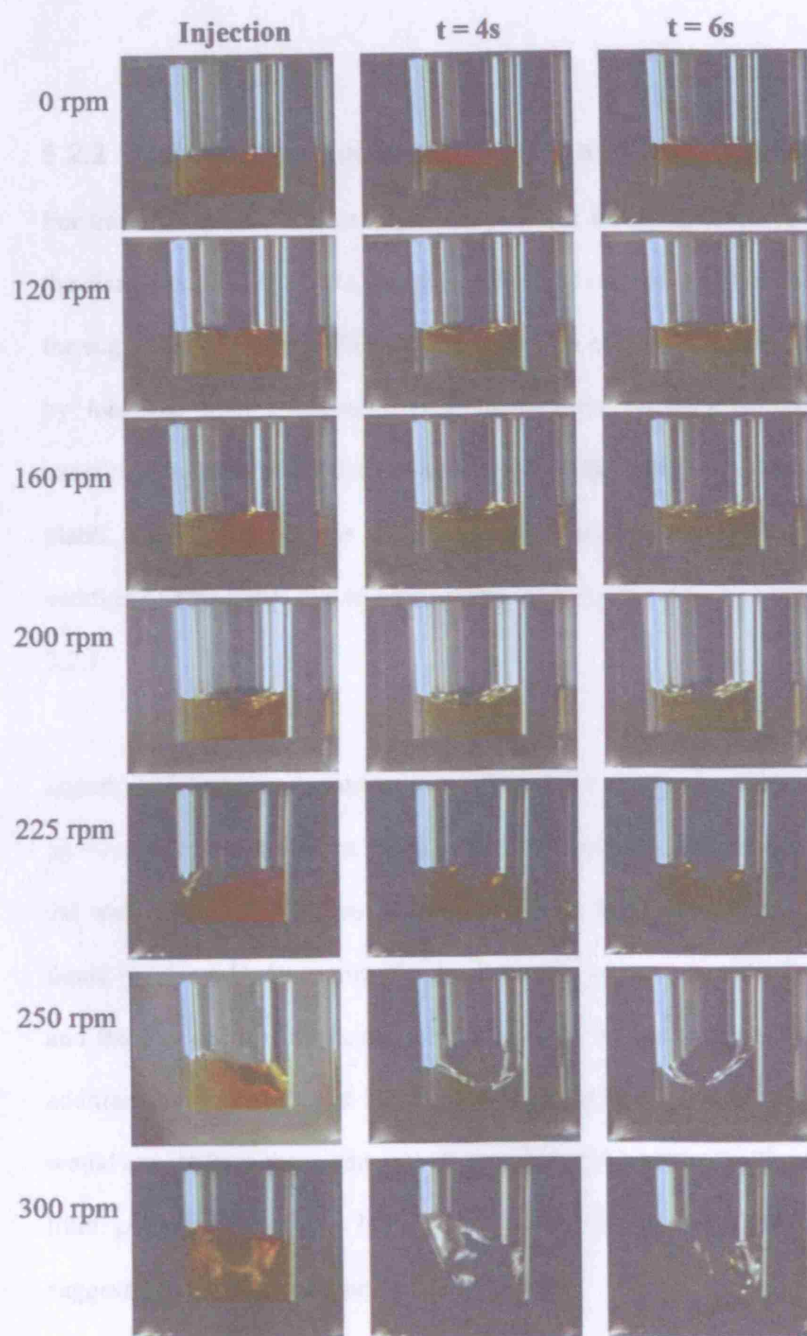


Figure 6.2. Still images from mixing time examination using 10% v/v liquid additions into 24-well ULA plates between 0-300 rpm, using iodine and sodium thiosulphate decolourisation. 100 μ l of 90 mM sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) solution was injected into 900 μ l 5mM Iodine (I_2) solution in an agitated well. Additions were performed with the 24-well ULA plate Perspex mimic using the mixing time addition rig described in Section 3.2.1. The microwell mimic was agitated on a Heidolph orbital shaker platform between 0-300 rpm. Images were captured with a high-speed camera described in Section 3.2.1, and processed using Adobe Photoshop CS2 (Uxbridge, UK).

6.2.2 Mixing pattern observations for additions to 24-well plates

For transient transfection in microwell plates, it would be useful to gain insight into the distribution of PEI-DNA complex solution during transfection. This is because of the suggested cytotoxic effects of PEI (Moghimi et al, 2005). This can be exacerbated by localized high concentrations of complexes. As such, mixing patterns were examined following the determination of mixing times for additions into 24-well plates. The mixing patterns of agitated microwell cultures were investigated by the addition of blue tracer dye to water in the 24-well plate mimic as described in Section 3.2.1.

Injection of the tracer dye under static (0 rpm) conditions showed that there was slight jet mixing (Figure 6.3). The force of the injection propelled the dye to the bottom of the well. There was also some retention of the dye on the surface meniscus of the liquid (Figure 6.3). Over time the dye is seen to spread out at the bottom of the well, and the dye diffuses upwards from the bottom, as was observed in Figure 6.2. For additions between 120 and 200 rpm, the mixing patterns were very similar, as one would expect from measurements of the mixing time shown in Figure 6.1. There was little perturbation of the liquid. The tracer dye shows trails moving laterally, suggesting that mixing occurs by radial motion.

As the shaking speed increases up to 200 rpm, the dye starts to form a conical shape at the bottom of the well. At 225 rpm, the dye at the bottom of the well starts to move. It was seen from the mixing times (Figure 6.1) that there is a change in the mixing regime between 200 rpm and 250 rpm. At 225 rpm, the liquid motion begins to lift the

dye off the bottom of the well. This suggests that there is not only radial mixing, but as the liquid hits the walls, axial mixing starts to take place as well. The image of dye addition at 6 seconds shows a lightly darker region remaining at the bottom of the well.

At 250 rpm and 300 rpm, there is large deformation of the liquid surface, same as the images observed in the decolourisation experiment (Figure 6.2). The dye no longer spreads out at the bottom of the well, as observed at lower shaking speeds, but takes a more vertical shape upon injection into the liquid. Strong movement of the liquid against the sides of the wall brings about axial mixing, which efficiently mixes the dye both axially and radially.

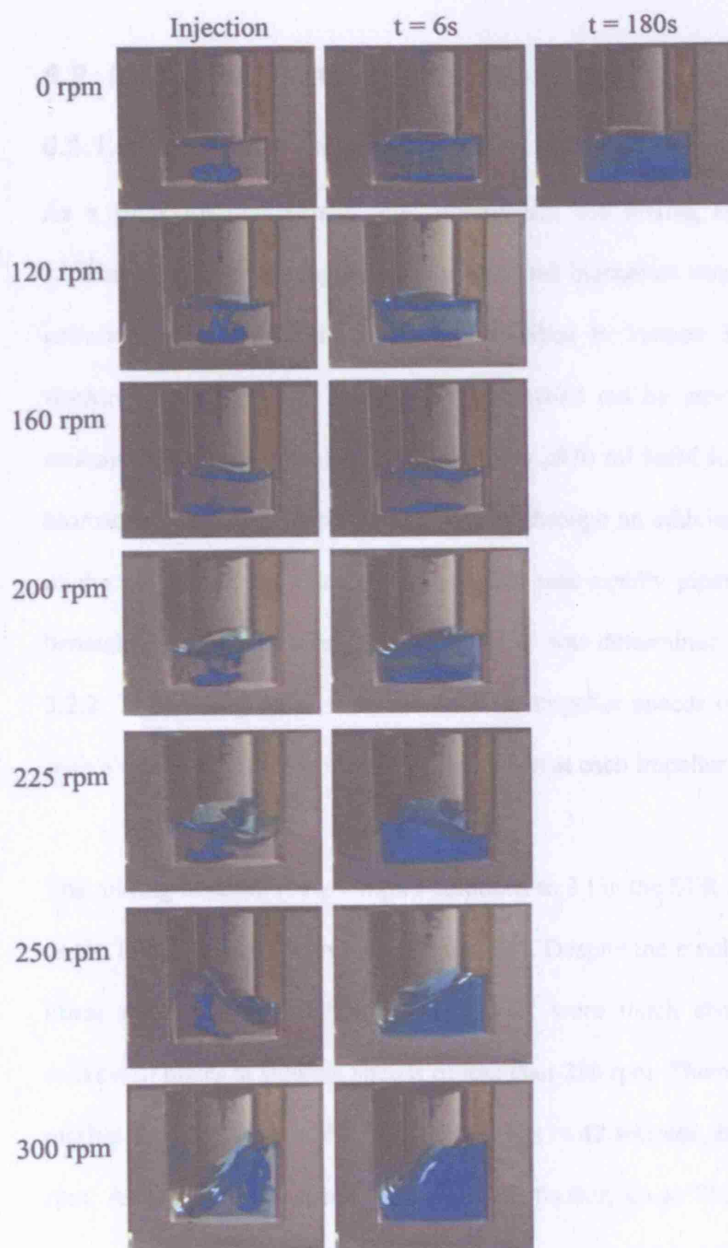


Figure 6.3. Still images from examination of mixing pattern using 1% v/v tracer dye liquid additions to 24-well ULA plates between 0-300 rpm. 10 μ l of blue food colouring tracer dye was added to 990 μ l of water in an agitated well. Additions were performed with the 24-well ULA plate Perspex mimic using the mixing time addition rig described in Section 3.2.1. The microwell mimic was agitated on a Heidolph orbital shaker platform between 0-300 rpm. Images were captured with a high-speed camera described in Section 3.2.1, and processed using Adobe Photoshop CS2 (Uxbridge, UK).

6.3 *Mixing in 5 l stirred tank bioreactor*

6.3.1 Mixing time measurements

As a basis for comparison with microwells, the mixing time for 1% v/v liquid additions to a 3 l (working volume) stirred tank bioreactor was also investigated. A 5 l unbaffled stirred tank bioreactor, as described in Section 3.1.7, was used with a working volume of 3 l. Additions were carried out by pipetting 30 ml of 0.99 M sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) solution into 2970 ml 5mM Iodine (I_2) solution in the bioreactor. The 25 ml pipette was inserted through an additions port on the top plate of the bioreactor, the thiosulphate solution was rapidly pipetted in with the tip just beneath the liquid surface. The mixing time was determined as described in Section 3.2.2. The mixing times were measure for impeller speeds of between 50-250 rpm, with a minimum of three measurements taken at each impeller speed.

The mixing time for 1% v/v liquid additions to 3 l in the STR decreased exponentially as the impeller speed increased (Figure 6.4). Despite the much larger volume, mixing times measured for additions to the STR were much shorter than those in the microwell plates at shaking speeds of less than 250 rpm. There was a large decrease in mixing time of almost 60%, from 99 seconds to 42 seconds, between 50 rpm and 100 rpm. As the impeller speed was increased further, up to 250 rpm, the mixing time approached a minimum. The difference between the mixing time at 200 rpm and 250 rpm was only 3 seconds; decolourisation was achieved within 8 seconds at 250 rpm.

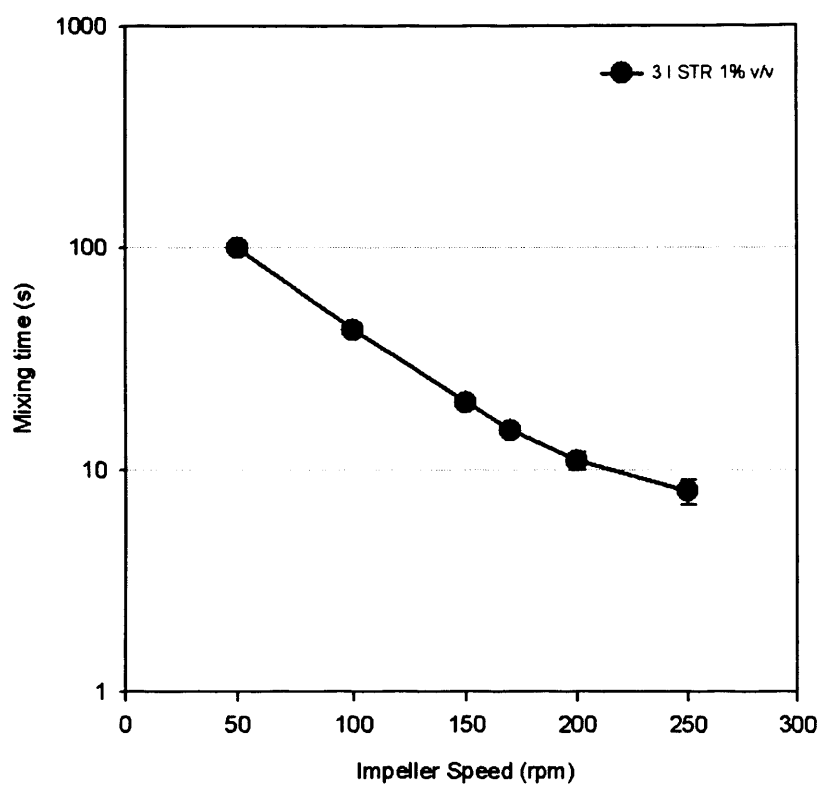


Figure 6.4. Liquid phase mixing time variation with increasing impeller speed measured in 5 l stirred tank reactor (STR) for 1% v/v liquid additions. A 3 l fill volume was used in a 5 l STR described in Section 3.1.7.1. 30 ml of 0.99 M sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) solution was added to 2970 ml 5 mM Iodine (I_2) solution in the STR as described in Section 3.1.7.2. Error bars represent one standard deviation about the mean of multiple measurements.

6.3.2 Mixing pattern observations for additions to 5L stirred tank bioreactor.

As outlined in Section 6.2.2, an understanding of the mixing patterns for additions of PEI-DNA complex solution would give insight into the effect of possible PEI cytotoxicity. In order to determine the distribution of complexes for additions to 5 l STR cultures, the mixing patterns were investigated by the 1% v/v liquid addition of blue tracer dye to 3 l of water in a 5 l STR.

From the still images in Figure 6.5, the additions to the liquid surface rapidly spread out laterally around the top of the liquid space. Even with the impeller inducing downward liquid motion, there was little initial axial spread of tracer dye. Dye was later drawn down through the impeller region, and then pushed towards the bottom of the vessel. There was a faint lighter region around the impeller region.

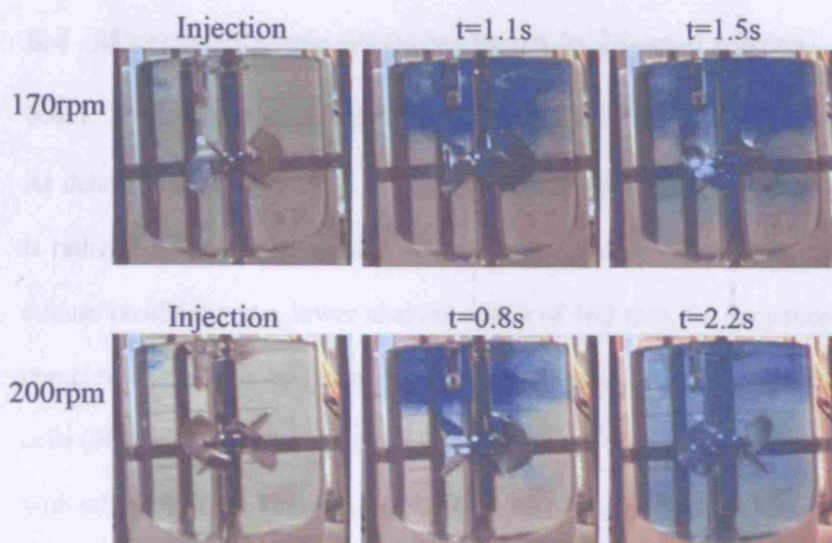


Figure 6.5. Still images from examination of mixing pattern using 1% v/v tracer dye liquid additions to the 5 l STR at impeller speeds of 170 rpm and 200 rpm. 30 ml of blue food colouring dye was added to 2970 ml of water in a 5 l STR as described in Section 3.1.7.1. Images were captured with a high-speed camera described in Section 3.2.1, and processed using Adobe Photoshop CS2 (Uxbridge, UK).

6.4 Microwell plate transfections in 24-well plates

6.4.1 Sequence of addition for transfections

As determined in Figure 6.1, the mixing time for additions to microwells at 160 rpm is rather long at 1460 seconds. In order to determine if this could be shortened for culture conditions at a lower shaking speed of 160 rpm, the sequence of addition of complexes and cells to the microwell was investigated. Adding a larger volume of cells (90% v/v) to the complexes (10% v/v) should provide better mixing as compared with addition of the complexes to cells. The 24-well ULA plates were transfected as described in Section 3.1.6. For the first plate, 900 μ l of CHO-S cells were seeded into each well, followed by the addition of 100 μ l of PEI-DNA complexes. In the second 24-well plate, complexes were added to each well first, followed by 900 μ l of CHO-S cells. The final volume in each well was 1 ml, with a cell density of 2.5×10^5 cells/ml, and a DNA concentration of 1 μ g/ml. Another 24-well plate was also seeded at 2.5×10^5 cells/ml as a negative untransfected control.

Cell growth kinetics for both transfected plates were similar (Figure 6.6). There was a slight initial drop in cell density post-transfection, to $1.2\text{--}1.5 \times 10^5$ cells/ml at 12 hours. The cell density recovered after 12 hours and reached 2.1×10^6 cells/ml at 72 hours for the plate where complexes were added to cells, and 2.2×10^6 cells/ml for the plate where cells were added to complexes. The slight initial drop in cell density was also observed for the untransfected control plate. The cell density for the negative control plate was similar to that of the transfected plates until 24 hours, but subsequently grew to much higher cell densities, reaching 4.3×10^6 cells/ml at 72 hours.

The transfection efficiency was higher in the microwell plate where the cells were added to complexes than the plate with complexes added to cells (Figure 6.7). In the plate where cells were added to complexes, the peak transfection efficiency of 29% GFP positive cells was measured at 48 hours. For the plate where complexes were added to cells, the peak transfection efficiency was observed at 24 hours, with 13% GFP positive cells.

The DNA concentration profiles post-transfection was quite similar over the culture period of 72 hours (Figure 6.8). The initial concentration measured in both transfections at 0 hours post-transfection was ~800 ng/ml in both cases. When the complexes were added to cells, the DNA concentration decreased faster than adding cells to complexes. In both microwell plate transfections, the DNA concentration decreased to around 50 ng/ml by 72 hours.

From Figure 6.7, adding the cells to complexes for transfections in a 24-well plate gives higher transfection efficiencies than adding complexes to cells. For subsequent microwell plate transfections, the PEI-DNA complexes were added to the wells first, followed by cells.

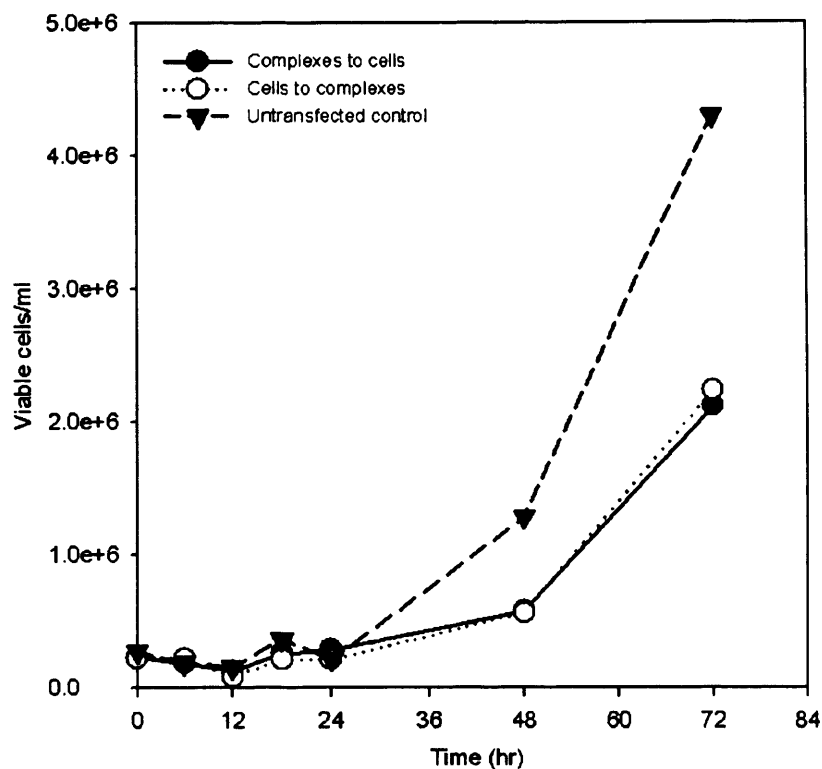


Figure 6.6 Growth kinetics of CHO-S cells transfected in 24-well plates; first plate with complexes added to cells, second plate with cells added to complexes. Cells were transfected in 24-well ULA plates as described in Section 3.1.6 at a shaking speed of 160 rpm. PEI-DNA complexes were formed with gWiz-GFP in 150 mM NaCl for 10 minutes as described in Section 3.1.4. Viable cell density was determined as described in 3.3.2. Viable cell counts performed as described in Section 3.3.2. Results show typical profiles from at least two replicate experiments.

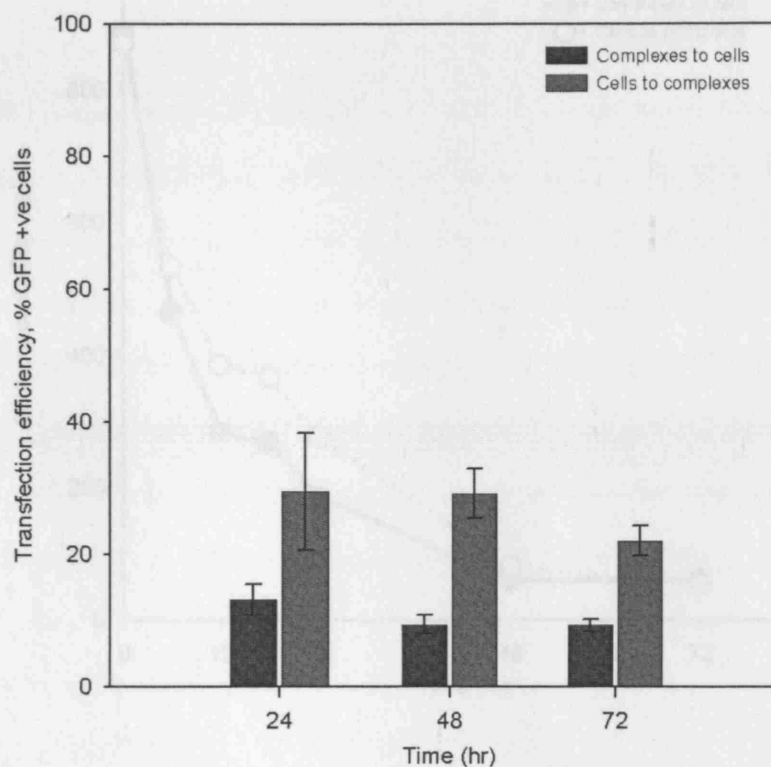


Figure 6.7 Transfection efficiency of CHO-S cells transfected in 24-well plates; first plate with complexes added to cells, second plate with cells added to complexes. Cells were transfected in 24-well ULA plates as described in Section 3.1.6 at a shaking speed of 160 rpm. PEI-DNA complexes were formed with gWiz-GFP in 150 mM NaCl for 10 minutes as described in Section 3.1.4. The number of GFP expressing cells was determined by flow cytometry as described in Section 3.3.5. Results show typical GFP expression levels from at least two replicate experiments. Error bars represent one standard deviation about the mean.

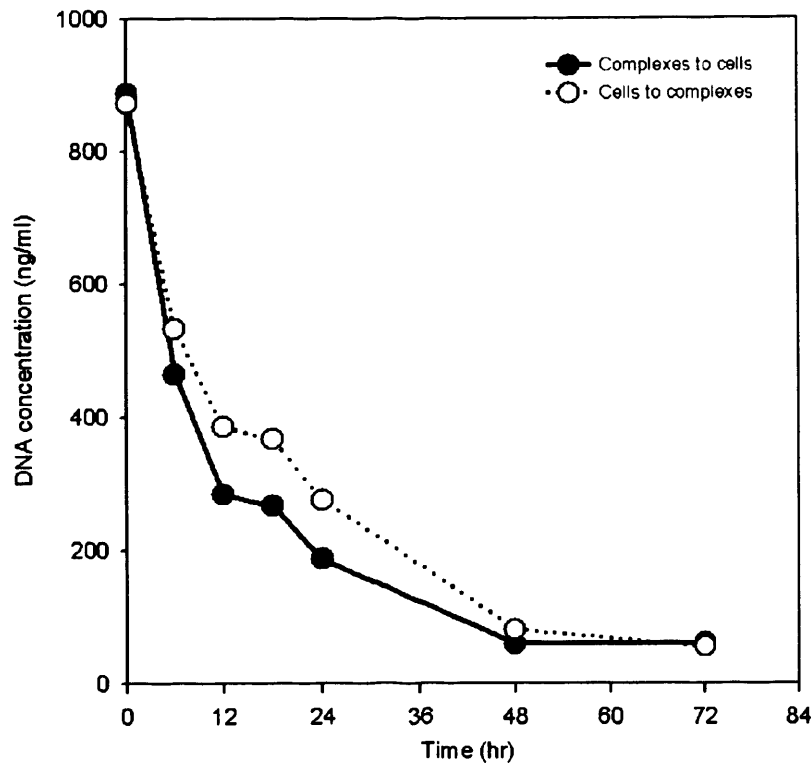


Figure 6.8 Concentration of DNA remaining in the supernatant post-transfection of CHO-S cells transfected in 24-well plates; first plate with complexes added to cells, second plate with cells added to complexes. Cells were transfected in 24-well ULA plates as described in Section 3.1.6 at a shaking speed of 160 rpm. PEI-DNA complexes were formed with gWiz-GFP in 150 mM NaCl for 10 minutes as described in Section 3.1.4. PEI-DNA complexes were added at time 0 hr. DNA concentration in the culture media was determined using with the Picogreen assay as described in Section 3.3.6. Results show averaged DNA concentrations from at least two replicate experiments.

6.4.2 Transfections at varying shaking speeds

Images from the mixing time experiments showed a large difference in the measured liquid phase mixing for additions to shaken microwell plates (Figure 6.3). As previously described (Section 6.2.2), there was a change in the mixing between 200 and 250 rpm, as the deformation on the liquid surface becomes increasingly pronounced. In order to ascertain the culture conditions for optimal transient transfection performance, transfections were carried out in 24-well ULA plates at various shaking speeds of 160, 200 and 250 rpm to investigate the effect these would have on transfections with PEI-DNA complexes. As was determined in Section 6.4.1, addition of cells to complexes was preferential for transfections in microwell plates. This procedure was carried out for the investigations into the effect of shaking speed. Transfections were carried out as described in Section 3.1.6.

The 24-well plate transfection at 200 rpm gave the highest cell density at 96 hours, of 2.6×10^6 cells/ml (Figure 6.9). The transfection at 250 rpm reached its peak cell density at 72 hours 1×10^6 cells/ml, then cell density decreased to 8×10^5 cells/ml at 96 hours. The slight dip in the cell density observed for transfections at 160rpm was not observed for transfections at 200 rpm or 250 rpm.

As shown in Figure 6.11, there did not appear to be a relationship between transfection efficiency and shaking speed for transfections in 24-well plates. The peak transfection efficiency was measured at 48 hours at all three shaking speeds, same as transfections in shake flasks. Transfections at 250 rpm had the highest measured transfection efficiency of 35.9% GFP positive cells. The 24-well plate transfection at

200 rpm had the lowest transfection efficiency of the three, with 25% GFP expressing cells measure at 48 hours.

The characteristic plateau in the DNA concentration between 0 hours and 24 hours post-transfection seen in transfections in shake flasks (Section 5.2) was not observed for the transfections in 24-well plates (Figure 6.11). The initial DNA concentrations measured immediately post-transfection were high, between 704 ng/ml for the transfection at 200 rpm and 887 ng/ml for the transfection at 160 rpm. The DNA concentration in all three transfections decreased to less than 100 ng/ml after 48 hours.

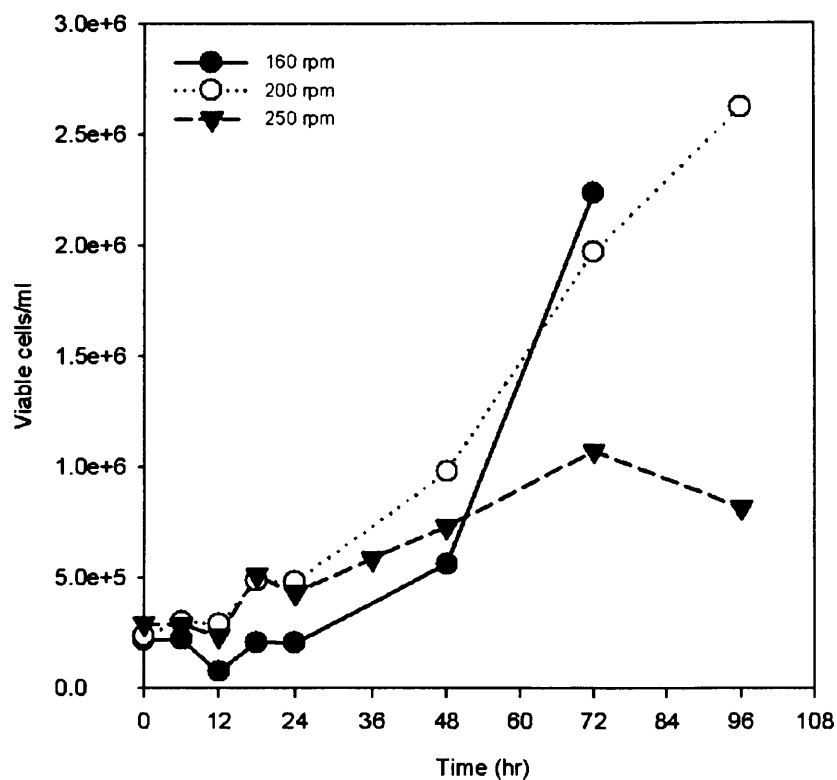


Figure 6.9 Growth kinetics of CHO-S cells transfected in 24-well plates at varying shaking speeds. Cells were transfected in 24-well ULA plates as described in Section 3.1.6 at shaking speeds of 160 rpm, 200 rpm and 250 rpm. PEI-DNA complexes were formed with gWiz-GFP in 150 mM NaCl for 10 minutes as described in Section 3.1.4. Viable cell density was determined as described in 3.3.2. Viable cell counts performed as described in Section 3.3.2. Results show typical profiles from at least two replicate experiments.

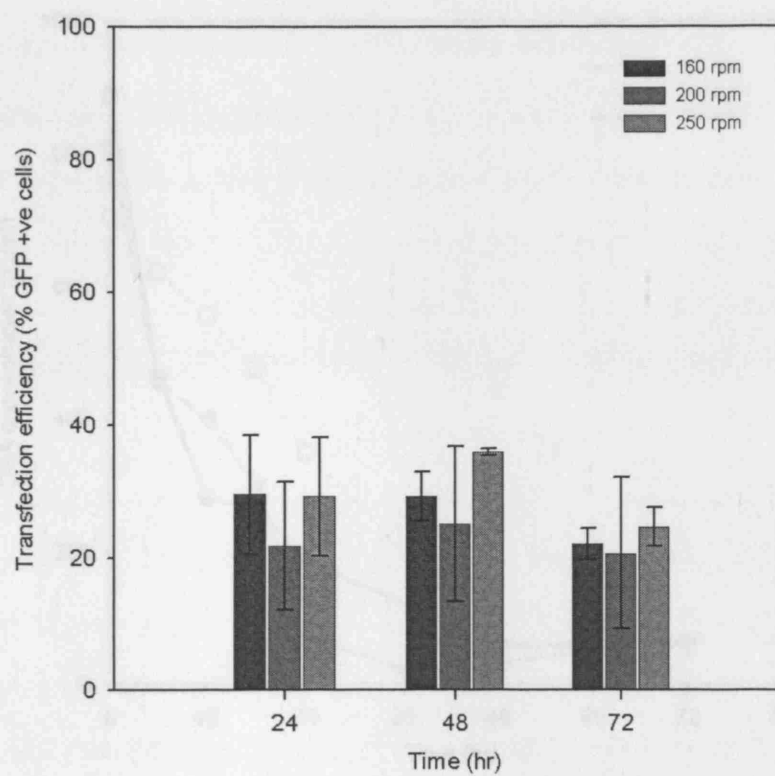


Figure 6.10 Transfection efficiency of CHO-S cells transfected in 24-well plates at varying shaking speeds. Cells were transfected in 24-well ULA plates as described in Section 3.1.6 at shaking speeds of 160 rpm, 200 rpm and 250 rpm. PEI-DNA complexes were formed with gWiz-GFP in 150 mM NaCl for 10 minutes as described in Section 3.1.4. The number of GFP expressing cells was determined by flow cytometry as described in Section 3.3.5. Results show typical GFP expression levels from at least two replicate experiments. Error bars represent one standard deviation about the mean.

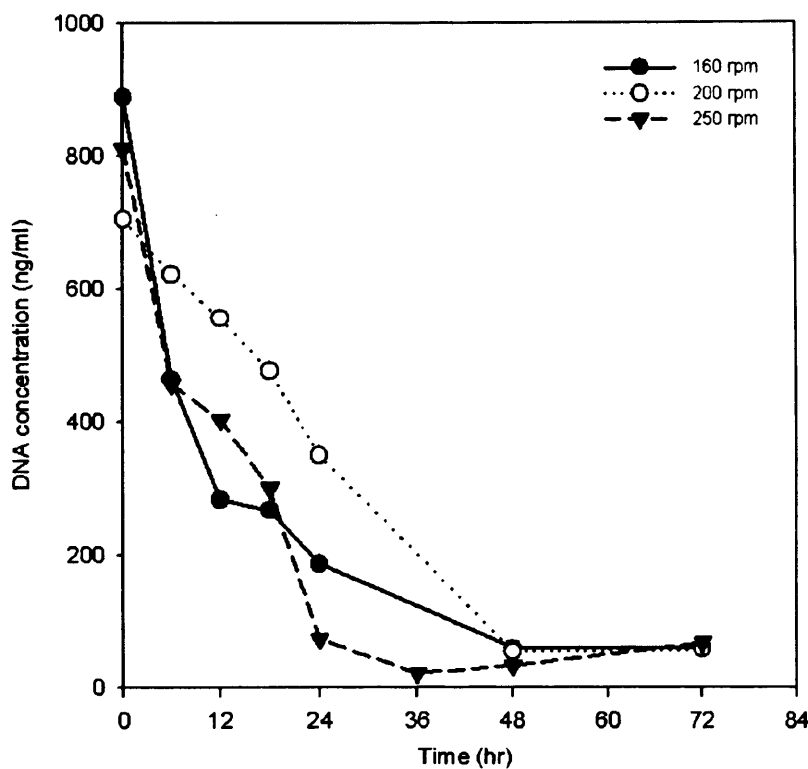


Figure 6.11 Concentration of DNA remaining in the supernatant post-transfection for CHO-S cells transfected in 24-well plates at varying shaking speeds. Cells were transfected in 24-well ULA plates as described in Section 3.1.6 at shaking speeds of 160 rpm, 200 rpm and 250 rpm. PEI-DNA complexes were formed with gWiz-GFP in 150 mM NaCl for 10 minutes as described in Section 3.1.4. PEI-DNA complexes were added at time 0 hr. DNA concentration in the culture media was determined using with the Picogreen assay as described in Section 3.3.6. Results show averaged DNA concentrations from at least two replicate experiments.

6.5 Transfections in 5 l stirred tank bioreactor

Transfections were also performed in a 3 l stirred tank bioreactor in order to investigate whether the transfection efficiencies observed in shake flasks (Section 5.2) and micro-well plates (Section 6.4) could be reproduced at a larger scale. The 5 l stirred tank bioreactor described in Section 3.1.7 was used for these experiments with a working volume of 3 l.

The bioreactor was set-up as described in Section 3.1.7. The bioreactor transfections were carried out as described in Section 3.1.7.4. The impeller speed was set to 150 rpm. Following results described in Section 5.4, 10% volume fraction of PEI-DNA complex solution was used to transfect the 3 l bioreactor. 300 ml of PEI-DNA complexes were aseptically prepared in a siliconised 500 ml glass bottle. 3 mg of gWiz-GFP plasmid DNA was diluted in 150 ml of 150mM NaCl. 4.17 mg of PEI 25kDa was diluted in 150 ml of 150 mM NaCl. Both solutions were rapidly mixed in the siliconised bottle and allowed to incubate for 10 minutes. The PEI-DNA complexes were then pumped into the bioreactor. Samples were taken at regular intervals and analysed as before.

At 24 hours post-transfection the cell density grew too high for headspace aeration to maintain the DOT level, and aeration was switched to intermittent sparging of air. Silicone antifoam A was added at 6 parts per million (ppm) to minimize the foaming. In addition to antifoam, the shear protectant Pluronic F-68 was added at 0.1% to further alleviate stress and damage to the cells (Murhammer and Goochee, 1990). Several experiments were initially performed in the bioreactor, and it was found that

downward liquid flow configuration lead to a large decrease in cell viability. The impeller was consequently switched to upward liquid flow, and this lead to an improvement in the survival of the cells.

The cell density in the bioreactor reached to 2.6×10^6 cells/ml (Figure 6.12) at 120 hours. At 96 hours, the cells density was 1.9×10^6 cells/ml, which is comparable to cell densities in 24-well plates and shake flasks. A drop in cell viability was measured between 24 to 48 hours; a corresponding decrease in the viable cell density was also measured over the same period. After 48 hours, the cells began growing again, and the viability started to improve slightly. A peak transfection efficiency of 58.3 % GFP positive cells was measured at 48 hours post transfection (Figure 6.13). This is comparable to transfection efficiencies observed in shake flask transfections with complexes formed in 150 mM NaCl (Figure 5.2a and Figure 5.7). The DNA concentration decreased up to 36 hours (Figure 6.14). After 48 hours post-transfection, with the decrease in cell viability from the sparging, the DNA release due to cell damage lead to interference of the assay and further accurate measurements were not possible.

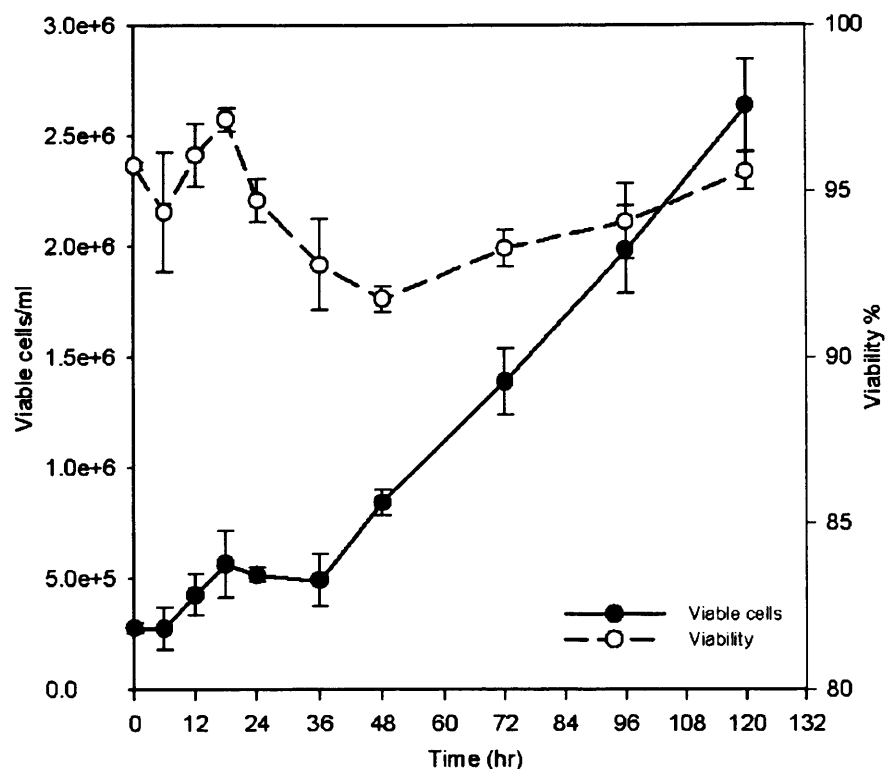


Figure 6.12 Typical growth kinetics of CHO-S cells from 5 l STR transfection with gWiz-GFP PEI-DNA complexes. Cells were transfected in a 5 l bioreactor with 3 l working volume as described in Section 3.1.7.3 and Section 3.1.7.4 at an impeller speed of 150 rpm. Complexes were prepared in 150mM NaCl for 10 minutes as described in Section 3.1.4. Viable cell density was determined as described in 3.3.2. Viable cell counts performed as described in Section 3.3.2. Error bars represent one standard deviation about the mean of at least three measurements.

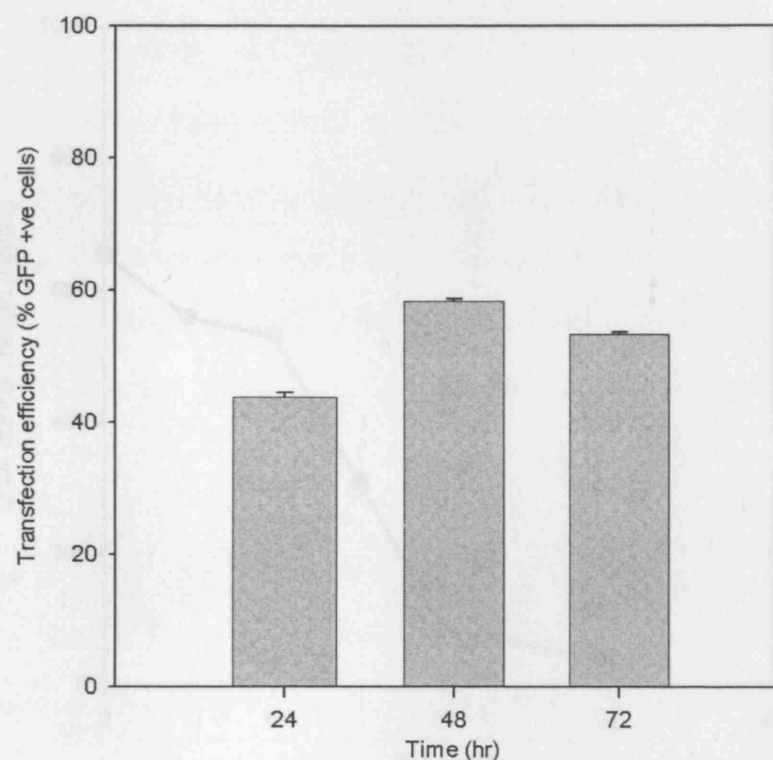


Figure 6.13 Typical transfection efficiency of CHO-S cells from 5 l STR transfection with gWiz-GFP PEI-DNA complexes. Cells were transfected in a 5 l bioreactor with 3 l working volume as described in Section 3.1.7.3 and Section 3.1.7.4 at an impeller speed of 150 rpm. Complexes were prepared in 150mM NaCl for 10 minutes as described in Section 3.1.4. The number of GFP expressing cells was determined by flow cytometry as described in Section 3.3.5. Error bars represent one standard deviation about the mean of at least three measurements.

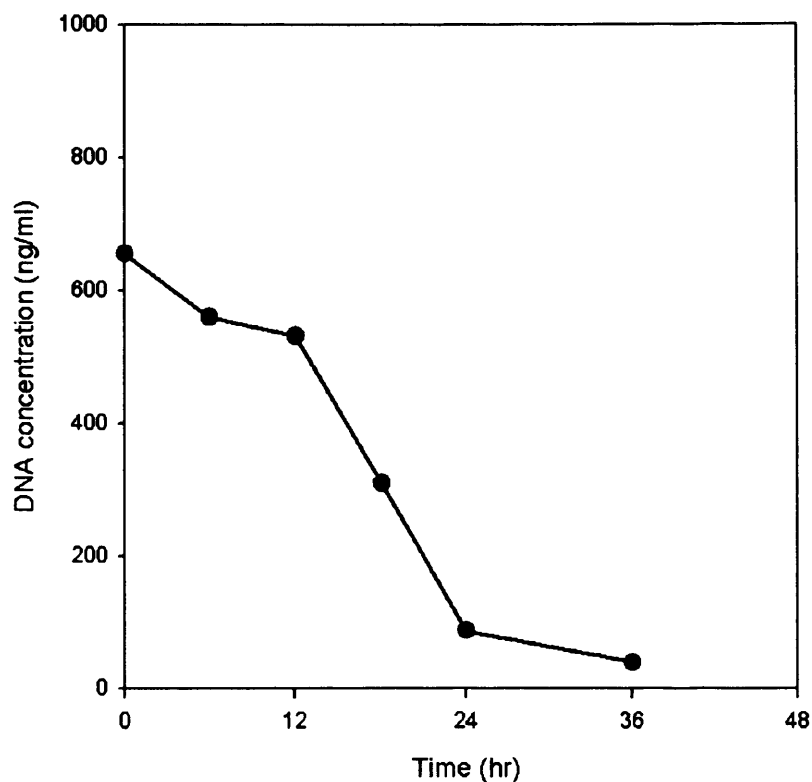


Figure 6.14 Typical Concentration of DNA remaining in the supernatant post-transfection for 5 l STR transfection of CHO-S cells with gWiz-GFP PEI-DNA complexes. Cells were transfected in a 5 l bioreactor with 3 l working volume as described in Section 3.1.7.3 and Section 3.1.7.4 at an impeller speed of 150 rpm. Complexes were prepared in 150mM NaCl for 10 minutes as described in Section 3.1.4. PEI-DNA complexes were added at time 0 hr. DNA concentration in the culture media was determined using with the Picogreen assay as described in Section 3.3.6. Results show averaged DNA concentrations from at least 3 measurements.

6.6 Comparison of recombinant protein production from transient transfection at different scales

After the individual investigations into transient transfection at each of the culture scales, a comparison of the productivity of transient transfection was carried out in each of the different scales. SEAP expression was used as an indication of secreted recombinant protein production. CHO-S cells were transfected in 24-well plates, 125 ml shake flasks and 5 l stirred tank bioreactor (3 l working volume) with PEI-DNA complexes formed with gWiz-SEAP plasmid DNA. From the mixing time experiments, the mixing time for the bioreactor at 70 rpm (Figure 6.4) is similar to mixing time at 150 rpm. Following the cell damage observed in the bioreactor transfection with PEI-DNA complexes formed with gWiz-GFP, the impeller speed was decreased to 70 rpm to further reduce the likelihood of cell damage. Once again, 24 hours post-transfection, headspace aeration was insufficient to maintain the DOT set point level of 30%, and aeration was changed to intermittent sparging of air at a flowrate of 100 ml/min (0.03 vvm). Silicone antifoam A was added at 6 parts per million (ppm) to minimize foaming from sparging.

At 96 hours, the highest cell density was measured in the transfection in the 24-well plate at 2.3×10^6 cells/ml. The 3 l bioreactor transfection had a cell density of 1.8×10^6 cells/ml at 96 hours (Figure 6.15), which is similar to the cell density measured at 96 hours for the bioreactor transfection with gWiz-GFP complexes (Figure 6.12). The cell density increased further in the bioreactor up to 2.5×10^6 cells/ml at 120 hours. There was a slight decrease in viability for the bioreactor transfection when aeration was turned to sparging at 24 hours (Figure 6.16), but the decrease was not as large as

that measured for the transfection with gWiz-GFP at 150 rpm (Figure 6.12). After 72 hours, the viability of the cells in the bioreactor started to drop off. The shake flask transfection only achieved a maximum cell density of 1.5×10^6 cells/ml, but viability remained high throughout; >95%.

The earliest expression of SEAP was measured in the 24-well plate transfection at 12 hours (Figure 6.17). The shake flask transfection followed this at 18 hours. SEAP expression was detected in the supernatant of the bioreactor transfection at 24 hours. At 96 hours, the SEAP expression levels across the three formats were all within the same range, from 2.4 mg/l in shake flask to 2.8 mg/ml in the 24-well plate, and 2.55 mg/l in the bioreactor. The SEAP concentration in the bioreactor dropped between 96 hours and 120 hours to 1.8 mg/l. This decrease in SEAP concentration corresponds to the drop in viability observed in Figure 6.16.

The decrease in DNA concentration profile was very similar across the three culture scales (Figure 6.18). In all three systems, the initial measurements of DNA concentration were around 600 ng/ml. The concentrations of DNA steadily decreased post-transfection from 6 hours, to less than 50 ng/ml by 36 hours in the 24-well plates, and by 48 hours in the shake flask transfections. The DNA concentration measured in the bioreactor transfection also decreased steadily up to 36 hours, but increased to 165 ng/ml at 48 hours. This increase in the DNA concentration is most likely related to the corresponding decrease in the viability for the bioreactor transfection shown in Figure 6.16. The increase in DNA concentration is most likely due to interference from the release of cellular DNA from damaged and dead cells (Alrubeai et al, 1995).

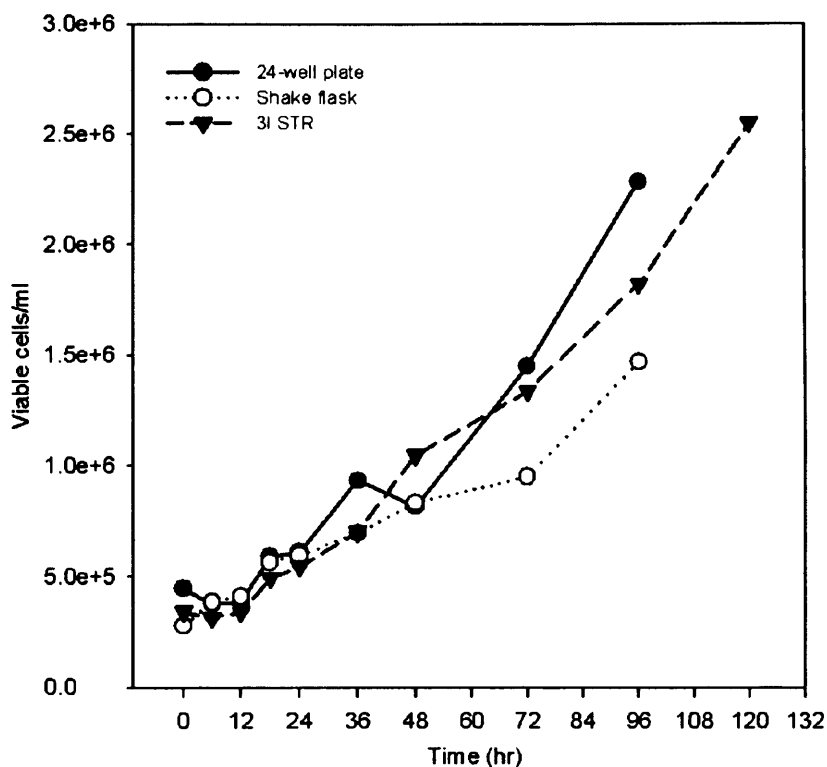


Figure 6.15 Typical growth kinetics of CHO-S cells from transfections with gWiz-SEAP PEI-DNA complexes in 24-well ULA plate, shake flask and 3 l STR. The 24-well plate was cultured (Section 3.1.6) at 250 rpm, and the shake flask was cultured at 130 rpm (Section 3.1.5). Bioreactor transfection carried out at 3l working volume (Section 3.1.7), at an impeller speed of 70 rpm. PEI-DNA complexes were prepared with gWiz-SEAP plasmid DNA in 150 mM NaCl for 10 minutes as described in Section 3.1.4. Viable cell counts performed as described in Section 3.3.2. Results show the average of at least 3 measurements.

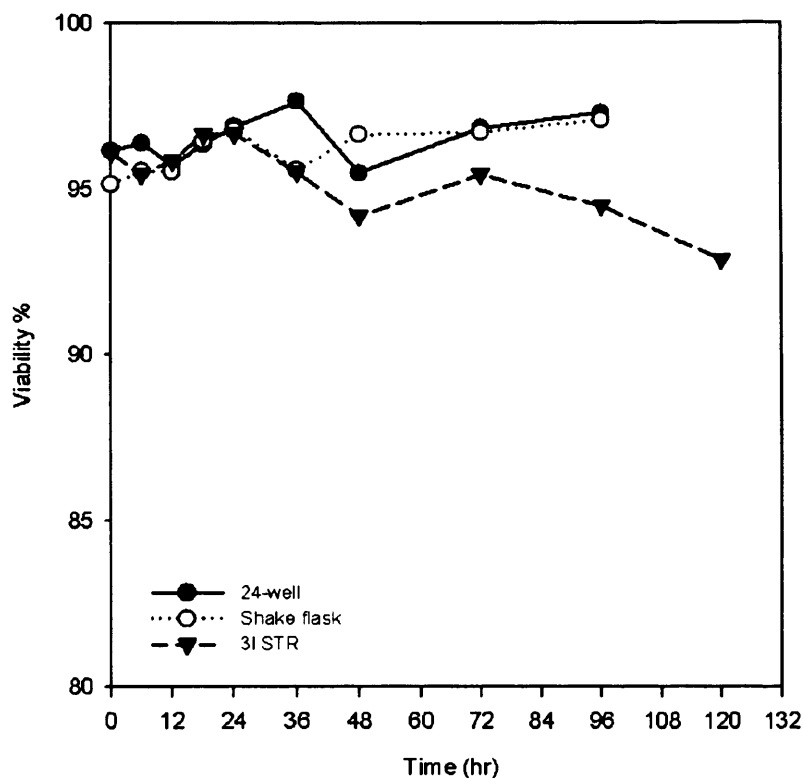


Figure 6.16 Cell viability during of CHO-S cells during transfections with gWiz-SEAP PEI-DNA complexes in 24-well ULA plate, shake flask and 3 l STR. The 24-well plate was cultured (Section 3.1.6) at 250 rpm, and the shake flask was cultured at 130 rpm (Section 3.1.5). Bioreactor transfection carried out at 3l working volume (Section 3.1.7), at an impeller speed of 70 rpm. PEI-DNA complexes were prepared with gWiz-SEAP plasmid DNA in 150 mM NaCl for 10 minutes as described in Section 3.1.4. Viability determined as described in Section 3.3.2. Results show the average of at least 3 measurements.

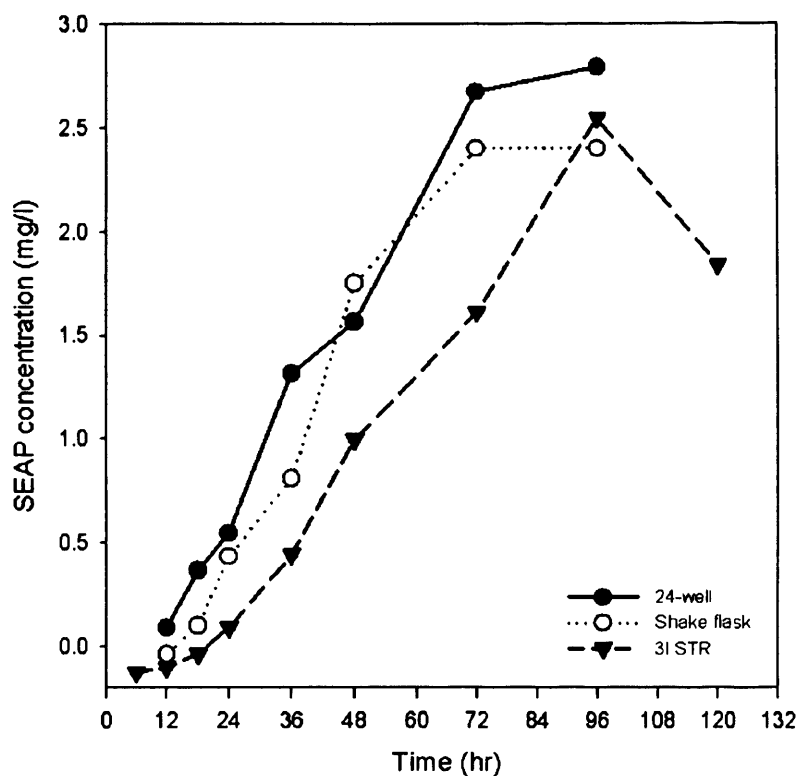


Figure 6.17 SEAP concentration in the supernatant for transfections of CHO-S cells with gWiz-SEAP PEI-DNA complexes in 24-well ULA plate, shake flask and 3 l STR. The 24-well plate was cultured (Section 3.1.6) at 250 rpm, and the shake flask was cultured at 130 rpm (Section 3.1.5). Bioreactor transfection carried out at 3l working volume (Section 3.1.7), at an impeller speed of 70 rpm. PEI-DNA complexes were prepared with gWiz-SEAP plasmid DNA in 150 mM NaCl for 10 minutes as described in Section 3.1.4. SEAP concentration was determined by the SEAP assay as described in Section 3.3.7. Results show the average of at least 3 measurements.

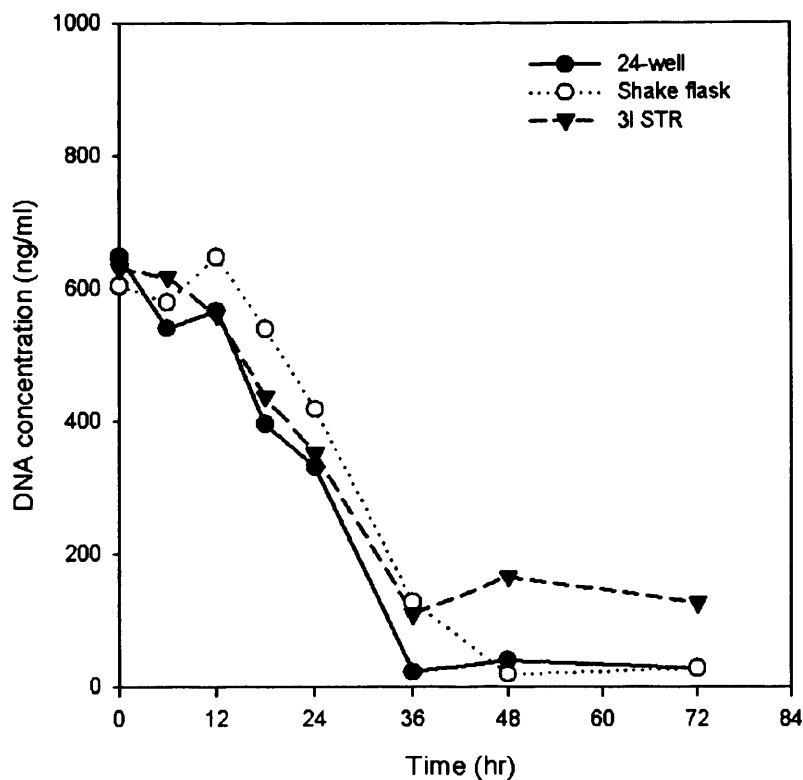


Figure 6.18 Concentration of DNA remaining in the supernatant post-transfection for transfections of CHO-S cells with gWiz-SEAP PEI-DNA complexes in 24-well ULA plate, shake flask and 3 l STR. The 24-well plate was cultured (Section 3.1.6) at 250 rpm, and the shake flask was cultured at 130 rpm (Section 3.1.5). Bioreactor transfection carried out at 3 l working volume (Section 3.1.7), at an impeller speed of 70 rpm. PEI-DNA complexes were prepared with gWiz-SEAP plasmid DNA in 150 mM NaCl for 10 minutes as described in Section 3.1.4. PEI-DNA complexes were added at time 0 hr. DNA concentration in the culture media was determined using with the Picogreen assay as described in Section 3.3.6. Results show averaged DNA concentrations from at least 3 measurements.

6.7 Discussion

Transient transfection can be a powerful tool in process development. But in order for it to be applied industrially, the procedure has to be scalable. Microwell plate technology has the potential for studying many process parameters in parallel. As such transient transfection, with its many process variables such as the N/P ratio and DNA concentrations, is an ideal candidate for the early application of microwell-based cell culture.

6.7.1 Mixing in microwells and bioreactors

The effect of additions to microwells and bioreactors at increasing shaking and impeller speeds were studied here by the use of the iodine and sodium thiosulphate decolourisation experiment and high-speed camera images. The clear decrease in mixing time between 200 rpm and 250 rpm for additions to microwell plates (Figure 6.1) corresponds to changes in the mixing regime observed in the still images from the high-speed camera (Figure 6.2 and 6.3). It seems that below 200 rpm, there was not enough energy put into the system to break the meniscus of the liquid (Figure 6.2). There was barely any vertical liquid movement, the surface of the liquid remained quite still, and mixing occurred predominantly by diffusive processes. Observations of mixing at shaking speeds around the transition in mixing time (225-250 rpm) suggest that lateral liquid movement forces the liquid up sides of the well, bringing about surface renewal (Figure 6.2 and Figure 6.3). This was evidenced by tracer dye moving sideways at the bottom of the well (Figure 6.3), and colourless regions flanking the brown region just below the liquid surface for additions at 225rpm seen in Figure 6.2. As the shaking speed increases up to 300 rpm, the throw

of the orbital shaker platform leads to the liquid moving up the sides of the well, and the deformation of the liquid surface actually starts to reach the bottom of the well (Figure 6.2). The tracer dye additions show vertical mixing as the liquid builds up at the walls (Figure 6.3). The sudden decrease in mixing times and increase in liquid surface deformation suggest a critical shaking speed is required to overcome the surface tension and liquid viscosity, before bulk liquid movement occurs.

In contrast, the bioreactor mixing time is comparatively shorter, despite the much larger volume involved (3 l as compared with 1 ml) (Figure 6.4). The axial mixing of the impeller is coupled with downward liquid flow from the impeller pitch. This combination of mixing leads to much faster dispersal of the additions throughout the bulk liquid volume. The downward liquid movement also forces the liquid against the round bottom of the bioreactor, encouraging lateral mixing up the sides of the vessel as seen in Figure 6.5 (170 rpm, $t = 1.5s$) with the blue regions to the bottom right and left of the impeller blades.

6.7.2 Transfections in microwells

The sequence of addition of complexes and cells to the microwell plate makes a difference to the transfection efficiency as seen from Figure 6.7. Cell growth profiles were very similar (Figure 6.6), but the transfection efficiency when adding cells to complexes in the well was almost three times higher than the transfection with the complexes added to the cells. As the cell growth was similar, it is not likely that the differences in transfection efficiency were due to PEI cytotoxicity (Fischer et al, 2003; Moghimi et al, 2005). It is most likely that the differences are due to the poor distribution of the complexes throughout the population of the cells when complexes

were added to cells. For additions of 10% v/v PEI-DNA complex solution at 160 rpm, the mixing time would be in the region of 24 minutes (Figure 6.1). The mixing patterns showed the additions would reside at the bottom of the well, slowly diffusing upwards over time (Figure 6.3), thus leading to more uneven distribution of complexes in the cell population and a lower transfection efficiency. The comparable trends in DNA concentration decrease over time could potentially be an artifact of the sampling process. As was suggested in Section 4.6.2, the complexes could bind rapidly on the available endocytosis sites on the cell surface (Rejman et al, 2005). During sampling, the pipetting would greatly enhance mixing and hence adherence of the complexes to the cell surface. This would mask any deficiencies in the DNA uptake due to poor mixing.

For investigations into the influence of shaking speed on microwell plate transfections, the 24-well plate agitated at 250 rpm had the highest transfection efficiency (Figure 6.10). There did not appear to be a correlation between shaking speed and measured transfection efficiency. As described in Section 6.7.1, adding cells to complexes could greatly enhance contact between cells and PEI-DNA complexes, reducing the dependence on agitation to bring about good mixing. The transition in mixing regime suggested in Section 6.7.1 could be responsible for the lower transfection efficiency for 24-well plate transfection at 200rpm. The lower cell density in the transfection at 250 rpm is most probably due to the higher transfection efficiency. The DNA concentration also decreased faster in the transfection at 250 rpm. This implies better mixing and more even distribution of the complexes, leading to faster uptake as the more of the endocytosis sites on the cell surface come into contact with the PEI-DNA complexes.

6.7.3 Transfections in bioreactors

Following the transfections in 24-well ULA plates, transfections in a 3 l STR were carried out to determine the scalability of transient transfection, scaling up 3000 times. There were initial difficulties with transfecting the CHO-S cells in the bioreactor. The impeller pitch with clockwise rotation was set to downward flow in an attempt to increase the residence time of bubbles in the system. With headspace aeration at the start of the culture, the cell viability remained high, and grew to 5×10^5 cells/ml by 24 hours. Gas transfer through the headspace was then unable to maintain the DOT set-point of 30%, and aeration was switched to intermittent sparging through the sintered porous metal sparger below the impeller. However, this was found to be detrimental to the cells, the viability dropping significantly after sparging was introduced. From previous work with VPM8 murine hybridoma cells, it was found that viability remained high with the impeller in an upflow configuration. The impeller pitch was consequently turned to the upflow configuration with clockwise rotation, and this seemed to reduce the extent of the viability decrease after aeration was turned to sparging at 24 hours (Figure 6.12). The combination of bubble damage from the sparging and the PEI cytotoxicity (Moghimi et al, 2005) is most likely to be responsible for the decrease in viability. It is known that small bubbles lead to damage of mammalian cells (Chisti, 1993; Chisti, 2000). This is coupled with the cells being cultured in serum free media, without the protective effects of serum (Chisti, 2000). Changing the impeller pitch for upwards flow would lead to shorter residence time of the bubbles, reducing the time available for the bubbles to induce damage to the cells. Even with the damage to the cells, the transfection efficiency for 3 l STR transfection was comparable to that measured in the shake flasks and the microwells. Although

there are other methods of aeration which do not subject the cells to damage from bubbles, such as the use of gas permeable membranes and an aeration cage, such methods are not implemented in large-scale processes, and sparging is the aeration method of choice (Hu and Aunins, 1997). It is conceivable that with the implementation of a suitable aeration system such as a ring sparger with larger bubbles, the cell viability would improve, leading to better productivity.

6.7.4 Protein productivity across different transfection scales

Mammalian cells are used for the production of biologically active secreted proteins. GFP as a reporter protein is useful to determine the percentage of cells that have received and are expressing the PEI-DNA complexes. But, as it is non-secreted, it is limited in giving an indication of likely protein productivity during the scale-up of transient transfection. For this, PEI-DNA complexes formed with gWiz-SEAP plasmid were used as described in Section 6.6. As the transient transfection process involves additions of the key component, PEI-DNA complexes, similar mixing times was used as a basis of comparison between transfections in microwells and bioreactors.

Transfections were performed on three scales, 24-well ULA plates, shake flasks and the 3 l stirred tank bioreactor. The SEAP was expressed at similar levels across the three platforms. Expression in the bioreactor was slower to start with as compared with the other two systems (Figure 6.17). This is most likely due to the additional stress on the cells from the impeller and sparging (Chisti, 1993). SEAP concentration dropped after 96 hours, which corresponded to the decrease in viability (Figure 6.16). Cell damage and cell death, leading to lysis, would release degradative proteases in to

the media environment (Alrubeai et al, 1995). The build-up of proteases would cause degradation of active SEAP proteins, leading to a decrease in the measured activity. The cell lysis arising from decreased viability would also lead to the release of cellular DNA into the media environment, accounting for the increase in DNA concentration after 48 hours in Figure 6.18.

SEAP productivity across the platforms are comparable to systems previously reported in other systems utilizing PEI-DNA complexes such as human embryonic kidney 293 cells (Schlaeger et al, 2003) and also CHO (Galbraith et al, 2006). Higher yields of up to 150 mg/L of recombinant proteins (Durocher et al, 2002; Derouazi et al, 2004; Baldi et al, 2005) have been reported for bioreactor systems utilizing novel modified transfection agents and cell lines. However, the scope of this work solely investigates PEI as a transfection agent in CHO-S cells. The current stirred tank bioreactor with direct batch addition of PEI-DNA complexes is the simplest system, and can rapidly test the potential of a transfection system without the need for too much process optimization. It is possible to improve current productivity by the use of chemicals such as microtubule disruptive agents (Tait et al, 2004). Another method used for large-scale transfections is the transfection at high cell density for several hours, followed by dilution with culture media up to the working volume (Derouazi et al, 2004). Transient transfection of HEK 293 cells combined with a perfusion bioreactor system have also proven to improve productivity (Sun et al, 2007). With further understanding into the physical mechanisms of microwells, coupled with additional process optimization, yields would most likely improve.

6.8 Summary

In this chapter it has been clearly shown that when studying transient transfection in a microwell format, the mixing of the system is an important consideration. With sub-optimal mixing, the maximum transfection efficiency that can be achieved is significantly reduced (Figure 6.7). At the low volumes employed in microwells, physical characteristics of the liquid such as surface tension and inertia exert a much greater effect. A critical shaking speed is required in order to overcome the inertial effects of surface tension and viscosity before true liquid movement and mixing is achieved in the bulk liquid. Further investigations showed that the highest transfection efficiency is obtained at 250 rpm for 24-well plates on the Heidolph orbital shaker platform. The transfection efficiency achieved using this shaking speed was found to be comparable to those observed in both shake flasks and bioreactors. At present, the application of transient transfection in shaken microwell plates is still in its early stages. With adequate characterization of the engineering environment, shaken 24-well plates can be used for initial development work on transient transfection.

From the studies into the scale up of transient transfection from microwell to bioreactor, similar SEAP protein productivities of 2.4-2.8 mg/l were achieved across the scales. This was despite the difficulties encountered during the transfections in the bioreactor. Here, the main focus has been on the characterization of PEI-DNA complexes. It has been demonstrated that the PEI-DNA complexes formed in 150 mM NaCl is a robust system that consistently gives good performance across different platforms. From the experiments of transient transfection investigating the protein productivity, with adequate characterization of the PEI-DNA complexes, culture conditions and the current choices of transfection agent (PEI), complex formation

buffer (150 mM NaCl), cell line (CHO) and culture media (serum-free), transient transfection can be a scalable process.

Chapter 7

7 Conclusions and Future Directions

7.1 Introduction

The investigations in this thesis have shown that transient transfection is a scalable process from microwell, to shake flask and bioreactor scales. But the scale-up also requires adequate characterization and understanding of the key parameters underpinning the interactions between PEI-DNA complexes, the environment during complex formation and transfection, and also the cells. The system of interactions between PEI-DNA complexes and the biophysical environment are complex. This work has primarily focused on the physicochemical characteristics of complexes formed in commonly used formation media and buffer, and to link them with the transfection performance in an industrially relevant context (CHO-S cells and serum-free media).

7.2 Physicochemical characterization of PEI-DNA complexes

The physicochemical characteristics of PEI-DNA complexes in different formation media were investigated. The performance of different complexes was studied by transfection of CHO-S cells. It was found that the key parameter influencing transfection efficiency is the zeta potential of PEI-DNA complexes during formation, which is in turn influenced by the aqueous environment in which the complexes are formed (Section 4.5). PEI-DNA complexes with strong surface charge zeta potentials give the highest transfection efficiency (Section 4.4). PEI-DNA complexes are dynamic structures, a decrease in concentration during transfection leads to a decrease

in size (Section 4.5.2). This was also shown when complexes were diluted in culture media, both during simulated (Section 4.3) and actual transfection (Section 4.5). The uptake of complexes during transient transfection is dependent on the culture media in which the transfection takes place (Section 4.5). The particle sizes of PEI-DNA complexes in different media, measured during formation, change during transfection (Section 4.3) and approach similar sizes when transfected in the same culture medium (Section 4.5). Thus, it was shown that characterisation of PEI-DNA complexes have to include studies during the transfection step, and not just the formation stage, in order to accurately describe their behaviour during transfection.

7.3 Process variables influencing transient transfection

From the investigations, the key process variables influencing transfection efficiency of PEI-DNA complexes were found to be the formation buffer, complex formation incubation time and the presence of conditioned media in the transfection environment. The transfection efficiency of PEI-DNA complexes formed in 150 mM NaCl is less sensitive to complex formation time than complexes formed in culture media such as CHO-S-SFM II and DMEM (Section 5.2). Increasing the length of formation incubation time leads to decreased transfection efficiency for complexes formed in these media (Section 5.2.2). Minimizing the presence of conditioned media gives better transfection efficiency for PEI-DNA complexes formed in 150 mM NaCl (Section 5.3). An increased proportion of conditioned media decreases transfection efficiency, and also increases the amount of variability in the process. In a large-scale process, a reduction in the volume fraction PEI-DNA complex solution would make it easier to handle and to implement process controls. The influence of volume fraction of PEI-DNA complexes was investigated (Section 5.4). It was determined that the

complexes formed in 10% volume fraction gives higher transfection efficiency than complexes formed in 5% volume fraction. Larger volumes of transfection solution used could lead to better distribution of complexes during transfection, giving higher transfection efficiency.

7.4 Scale-up of transient transfection: from microwell plate to bioreactor scales

The scalability of transient transfection was investigated by comparing transfection efficiency and recombinant protein production in 24-well ultra-low attachment plates, shake flasks and a stirred tank bioreactor. Investigations into mixing time over several shaking speeds for liquid additions to 24-well plates showed that a critical speed is required for the inertial effects and surface tension to be overcome, in order to induce mixing in the bulk liquid (Section 6.2). The mixing time in the 3 l stirred tank bioreactor was, by comparison, shorter than that of the 24-well plate. High-speed camera still images showed that there was good lateral and axial mixing achieved with the 3-segment pitched blade impeller, even at low impeller speeds (Section 6.3). Transient transfection conditions were found to be scalable; the transfection efficiency was comparable across the three geometries. Microwell plate transfections showed that mixing is important, with better transfection at faster shaking speeds (Section 6.4). Transient transfection conditions were scaled-up from the microwell to the bioreactor. Secreted protein productivity was investigated by measuring SEAP expression levels from the three scales; 24-well ultra-low attachment plates, shake flasks and 3 l bioreactor. The similar SEAP expression levels of 2.4-2.8 mg/l were obtained across the three scales (Section 6.6). The recombinant protein productivity is comparable to that reported in other systems.

7.5 Future directions

7.5.1 PEI-DNA complexes

This thesis examined the use of 25kDa branched PEI polymer as a condensation agent for the delivery of DNA for recombinant protein production. It was determined that the uptake of PEI-DNA complexes is not the barrier for transgene expression. Rather, gene expression is governed by intracellular mechanisms (Section 4.4).

Future work could investigate the potential for improvement of gene delivery ability of complexes to the nucleus in order to increase protein productivity. Addition of nuclear localization sequences can help direct the transgene into the nucleolus for protein expression (Chan and Jans, 1999; Nagasaki et al, 2003; Medina-Kauwe et al, 2005).

Modification of PEI by conjugation in order to improve delivery is also a possible option for process improvement (Ogris et al, 1998; Merdan et al, 2005; Lungwitz et al, 2005). PEI is commonly used because it is a simple and affordable agent for transient gene expression. In order for modified PEIs to be accepted industrially, they first have to be widely available, and also cost-effective. Large-scale transfections necessitate the use of large amounts of complexation agent; cost then becomes an increasing consideration, as scales grow larger.

Another approach for improvement of the PEI-DNA complexes would be to modify the complex formation procedure and environment. It has been found that a

combination of small and medium sized PEIs can improve packing of the complexes, enhancing the transfection efficiency (Godbey et al, 1999a).

It was also determined that strongly positive surface charge during complexation was linked to improve transfection efficiency (Section 4.4). Future work could investigate improvement of the complex formation for strong zeta potential, as such this might involve investigating other more innovative types of formation media, or the use of additives.

7.5.2 Transient transfection conditions

The industrial relevance of this work has been a prime consideration when investigating the conditions for transient transfection. Therefore, a straightforward procedure for transient transfection was been adopted for the basis of these experiments.

A method commonly employed for transient transfection is to incubate cells with “transfection media” containing PEI-DNA complexes for a period of time, typically 4 hours, then either diluting out the transfection media or changing the media completely to growth media (Girard et al, 2001; Wightman et al, 2001; Girard et al, 2002; Derouazi et al, 2004). This is primarily used in instances where serum is required for cell growth. The advantage of such a method is the cells are not exposed to the cytotoxic effects of PEI for a prolonged period of time. And as such a higher concentration of PEI-DNA complexes can be used without fear of detriment to the cells. Varying the cell seeding density prior to transfection is a possible means for improving the process. A higher cell concentration during transfection would lead to

increased probability of contact between cells and complexes, increasing the initial amount of complexes bound to endocytosis sites on the cell surface.

The use of additives is a potential method for increasing transfection efficiency. These agents are predominantly used to affect the interaction between cells and complexes post-transfection. Microtubule disruptive agents and bovine serum albumin (BSA) have been shown to improve transfection efficiency in CHO-S cells (Tait et al, 2004).

The measurement of DNA concentration in the supernatant suggests that complexes are commonly endocytosed by the cells by 48 hours post-transfection. Further addition of PEI-DNA complexes once the initial concentration of complexes has been taken up could possibly improve protein production. Such an approach is similar to the fed-batch strategy commonly employed in the encouragement of increased productivity in animal cells (Zhou et al, 1997; Hu and Aunins, 1997; Mori et al, 2004; Sun et al, 2006), except in this case the substrate would be DNA containing the transgene. A consideration is the higher concentrations of PEI in the culture media, if further additions of PEI-DNA complexes are made. This might necessitate a method for removal of at least a portion of excess PEI to mitigate potential cytotoxicity.

7.5.3 Application of transient transfection to industrial processes

There can be further improvement to the production of recombinant proteins via transient transfection in bioreactors. The usual factors underpinning “conventional” cultivation of animal cells in reactors are also relevant to the implementation of transient transfection at an industrial scale (Chisti, 1993; Hu and Aunins, 1997; Kretzmer, 2002; Langheinrich et al, 2002). Furthermore, the cells are exposed to the

possible cytotoxic effects of PEI, thus requiring a more careful examination of factors that could lead to potential cell damage. Methods, such as perfusion or some form of online membrane separation, could be employed to remove the excess PEI from the cell environment once it has been determined that the complexes have been taken up by the cells in order to reduce the likelihood of cell damage from prolonged exposure of PEI.

Transient transfection can be used to rapidly obtain recombinant proteins within a matter of days (Wurm and Bernard, 2001). During drug development, transient transfection enables several variables across the recombinant protein product to be studied in a short amount of time. During selection process for production cell lines and expression vectors, the protein product gene can be cloned into several potential expression systems and simultaneously investigated. This enables the optimal combination of cell line, expression levels, and protein integrity to be selected. It is foreseeable to use microwell plates for the screening of different expression plasmids for transfection efficiency and productivity. On a 24-well plate, several plasmids could be investigated at the same time, with each plasmid studied in replicate in two to three wells. In this manner, up to 12 plasmids can be measured on each plate, greatly enhancing the throughput of screening work. Productivities can be measured at a particular time point, thus transient transfection can be a very powerful process development tool, especially if used in conjunction with high throughput platforms such as microwell plates.

From the work that has been presented here, in order to develop a transient transfection process for the production of recombinant proteins the following optimization steps should be implemented. Firstly, the formation buffer for the PEI-

DNA complexes should be selected in conjunction with variables such as the N/P ratio and the culture media, so as to obtain complexes with strong zeta potentials during formation. These can be carried out using high-throughput methods that allow for rapid determination of complex formation conditions. Following which, microwell plate cell culture can be used to determine the best set of process variables described in Chapter 5, such as the complex formation time, as well as culture conditions in order to optimise the yield. These rapid tools can be utilised for efficient transient transfection process development when looking to industrial application.

In conclusion, with adequate consideration of the physicochemical characteristics of transfection complexes and the process parameters, transient transfection has been demonstrated in this work to be a scalable process that is suitable for application at the industrial scale for the rapid generation of recombinant proteins.

Appendix A: Evaporation in agitated 24-well ULA plate cultures

The water loss from evaporation in each well was monitored by measuring the weight of each sample in a microcentrifuge tube of predetermined weight. 24-well plates were sealed with the BreatheEasy membrane and placed in a humidified CO₂ incubator as described in 3.1.6. The sample weight is difference between the total weight and the weight of the microcentrifuge tube. The fill volume used in each well was 1 ml, which corresponds to 1.0 g of culture in each well. Losses in sample weight were attributed to water loss through evaporation. A series of experiments was performed to investigate the amount of water loss over the course of a culture. Figure A.1 shows the extent of water loss from shaken 24-well plates

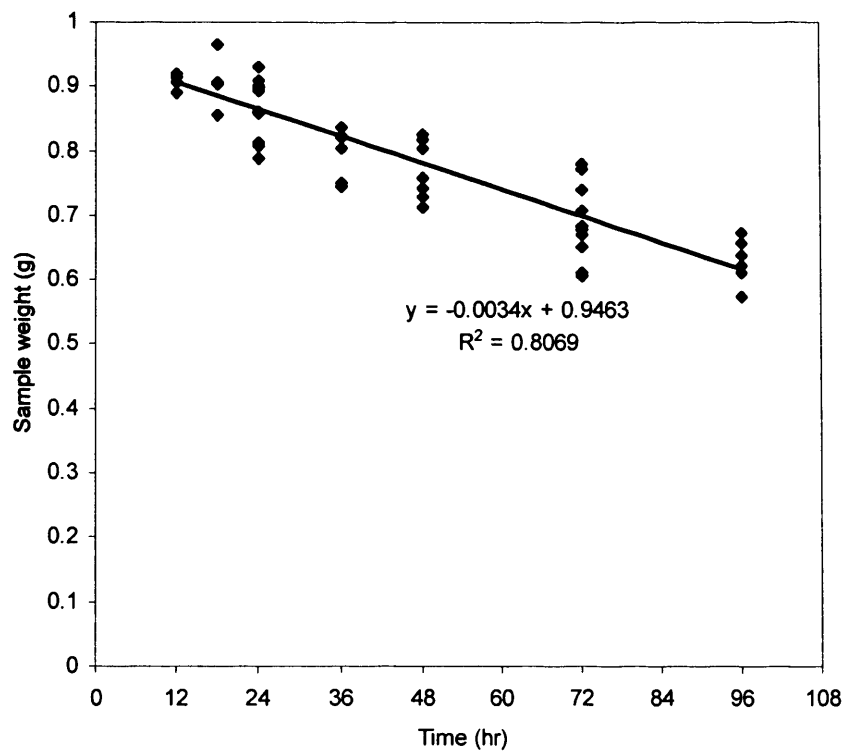


Figure A.1 Evaporation from shaken 24-well plates sealed with BreatheEasy membrane.

Appendix B: Development of modified Picogreen™ assay with heparin mediated dissociation of PEI-DNA complexes

In addition to the use of the Zetasizer 3000 to measure the cellular uptake of the PEI-DNA complex particles, this assay was developed to measure the remaining plasmid DNA concentration in the culture media correlate this with the uptake of complex particles. It was previously reported that heparin at concentrations above 1 mg/ml induces the dissociation of PEI DNA complexes (Moret et al, 2001).

Here, PEI-DNA complexes were prepared with known concentrations of plasmid DNA as described in Section 3.1.4 and serially diluted in cell culture media to form a standard curve. Heparin was then added at a final concentration of 1mg/ml to cause complex dissociation in order for Picogreen™ to access the plasmid DNA. Controls were also prepared. As a positive control free plasmid DNA without PEI was diluted in cell culture media, followed by the addition of heparin and Picogreen™. PBS was added to complexes instead of heparin as a negative control to show that heparin causes complex dissociation, followed by Picogreen™. From the results shown in Figure B.1, it was seen that the Picogreen™ fluorescence signal detected when heparin was added to complexes was identical to that observed when plasmid DNA alone was used. In the absence of heparin, increase in signal was not detected, even with increasing concentrations of plasmid DNA in the complexes. This implies that, plasmid DNA is inaccessible to Picogreen™ when complexed with PEI molecules. With the addition of heparin, dissociation of the complexes occurs, allowing Picogreen™ to intercalate into the freed plasmid DNA. In this way, standard curves of

plasmid DNA in PEI complexes can be obtained, allowing the quantification of complex plasmid DNA remaining in post-transfection cell culture media.

The preparation of the standard curves of heparin dissociated PEI-DNA complexes were further fine-tuned by adjustment of the plate reader settings. Figure B.2 shows a typical standard curve for the measurement of DNA concentration remaining in the supernatant post-transfection.

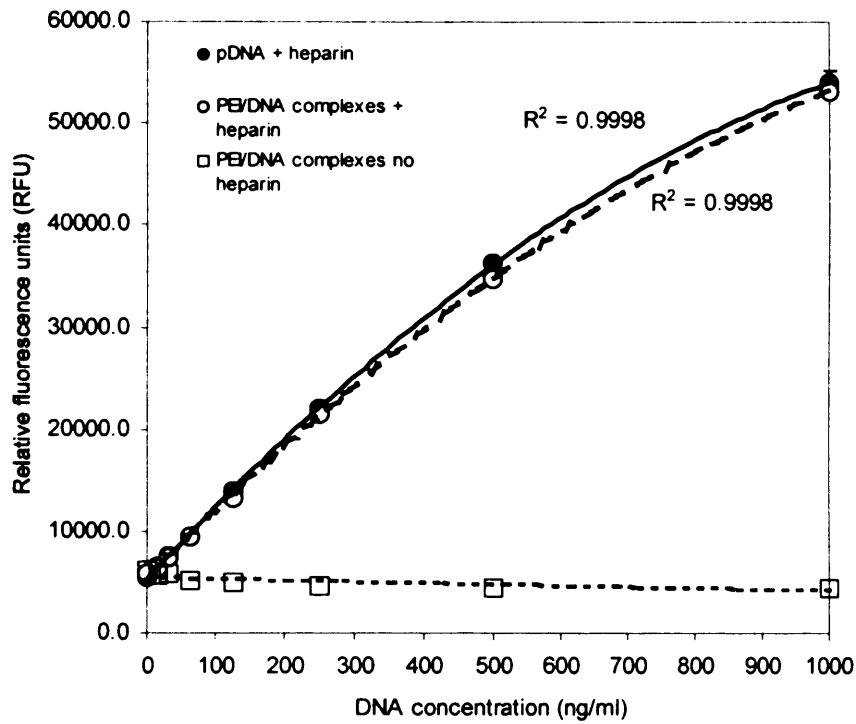


Figure B.1 Picogreen™ quantification of DNA for heparin dissociated PEI-DNA complexes. Complexes were prepared in CHO-S-SFM II cell culture media as described in Section 3.1.4. The complexes serially diluted in CHO-S-SFM II media from 1 $\mu\text{g/ml}$ to 0.016 $\mu\text{g/ml}$. Heparin was added to each well, at a concentration of 3 mg/ml .

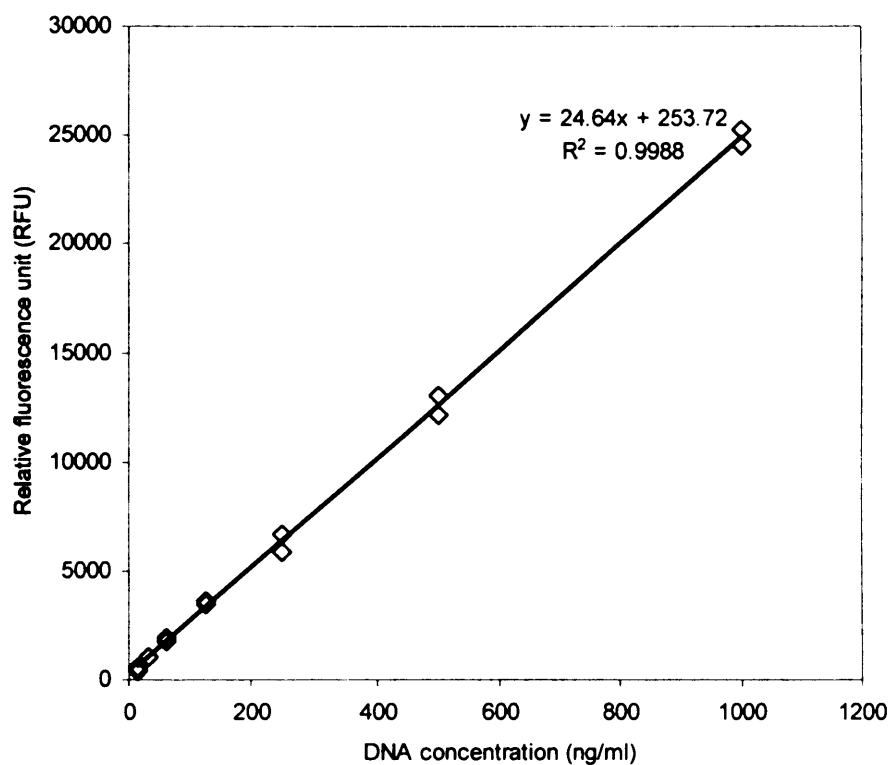


Figure B.2 Typical standard curve for Picogreen™ to determine of DNA concentration remaining in the supernatant post-transfection. Complexes were prepared in CHO-S-SFM II cell culture media as described in Section 3.1.4. The complexes serially diluted in CHO-S-SFM II media from 1 µg/ml to 0.016 µg/ml. The Picogreen assay was performed as described in Section 3.3.6.

Appendix C: Composition of Dulbecco's Modified Eagles

Medium

Component	Concentration (g/l)
<u>Inorganic salts</u>	
CaCl ₂ .2H ₂ O	0.265
Fe(NO ₃) ₃ .9H ₂ O	0.0001
MgSO ₄ (anhydrous)	0.09767
KCl	0.4
NaHCO ₃	0.37
NaCl	6.4
Na ₂ HPO ₄ (anhydrous)	0.109
<u>Amino acids</u>	
L-Arginine.HCl	0.084
L-Cysteine.2HCl	0.0626
L-Glutamine	0.584
Glycine	0.03
L-Histidine.HCl.monohydrate	0.042
L-Isoleucine	0.105
L-Leucine	0.105
L-Lysine.HCl	0.146
L-Methionine	0.03
L-Phenylalanine	0.066
L-Serine	0.042
L-Threonine	0.095
L-Tryptophan	0.016
L-Tyrosine.2Na.dihydrate	0.10379
L-Valine	0.094
<u>Vitamins</u>	
Choline chloride	0.004
Folic acid	0.004
myo-Inositol	0.0072
Niacinamide	0.004
D-Pantothenic acid.1/2Ca	0.004
Pyroxidine.HCl	0.004
Riboflavin	0.0004
Thiamine.HCl	0.004
<u>Sugars</u>	
D-Glucose	4.5
<u>Other</u>	
Phenol red.Na	0.0159
Pyruvic acid.Na	0.11

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